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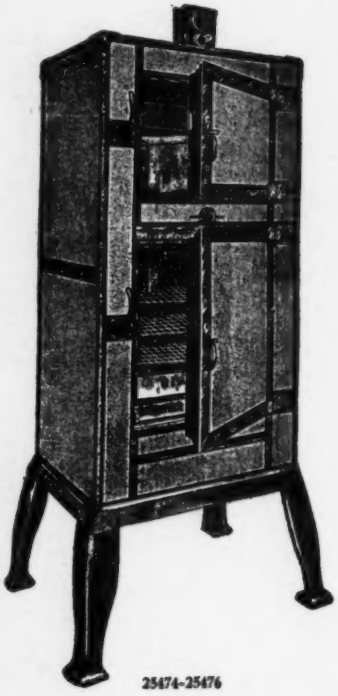

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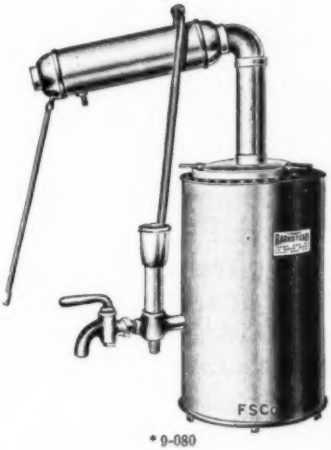

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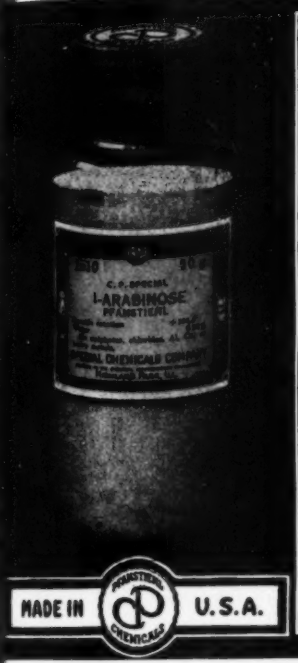

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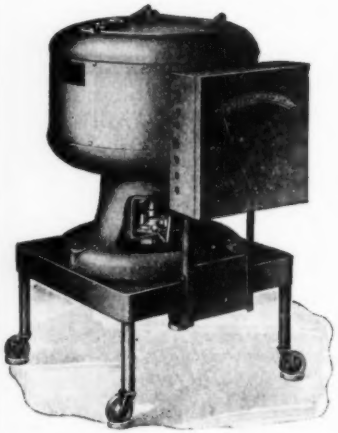

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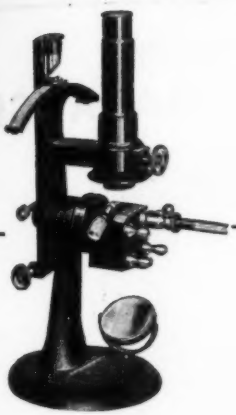

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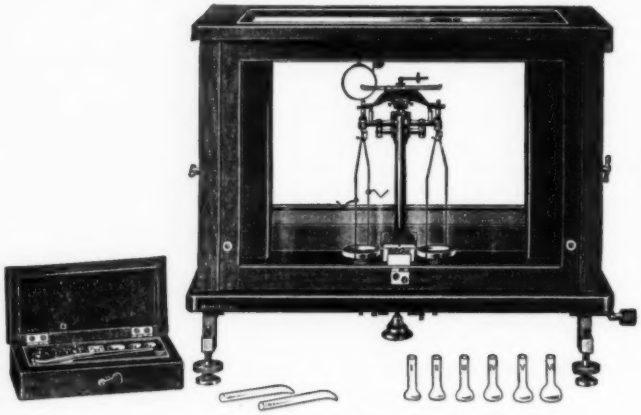

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THE FUNDAMENTAL FOOD REQUIREMENTS FOR THE GROWTH OF THE RAT.

III. YEAST AND YEAST FRACTIONS AS A SUPPLEMENT TO SYNTHETIC RATIONS.*

By CORNELIA KENNEDY and LEROY S. PALMER.

(From the Section of Animal Nutrition, Division of Agricultural Biochemistry, University of Minnesota, St. Paul.)

(Received for publication, October 21, 1927.)

In a former paper (1) we have discussed yeast as a source of vitamin B and have shown that under the conditions in which the experimental work was then carried out, yeast was not as good a source of this factor as the alcoholic extract of wheat embryo. We based this conclusion on the fact that it took larger amounts of dried yeast to attain satisfactory growth than it took of the alcoholic extract of the embryo, and that thereby a greater amount of unknown substances, including those of nitrogenous nature, was added to a purified ration.

Recently (2) we have shown that as we refined our laboratory technique, we met with greater and greater difficulty in formulating a purified basal ration which satisfied the requirements of growth. Of the many additions we made to our basal rations in our attempts to determine what was lacking, yeast proved to be one of the few substances which added something to the ration that was necessary for growth.

The data reported in this paper include (1) a study of starch-free dry yeast,[1] and various preparations from this yeast as sources of vitamin B, and (2) a study of the same yeast and the various preparations as supplements to our basal ration.

* Published with the approval of the Director, as Paper No. 728, Journal Series, Minnesota Agricultural Experiment Station.

[1] This yeast was purchased from the Northwestern Yeast Company, Chicago.

EXPERIMENTAL.

Yeast and Yeast Preparations as Sources of Vitamin B.—Our general laboratory technique and preparation of food materials has already been described in detail in a previous paper (2). In all the experiments described in this paper the rats were kept in colonies on 3 mesh per inch wire screens raised 1 inch above the floor of the cage. In Lot 1, Chart 1, of this series, young rats were placed on experiment when 4 weeks old and were fed a basal ration consisting of casein (purified by our method) 18 parts, salts (3) 3.7 parts, agar 2 parts, butter fat 5 parts, Crisco 10 parts, and dextrin 61.3 parts. The yeast was fed separately as a 0.5 gm. pellet per rat per day. From an inspection of the growth curve

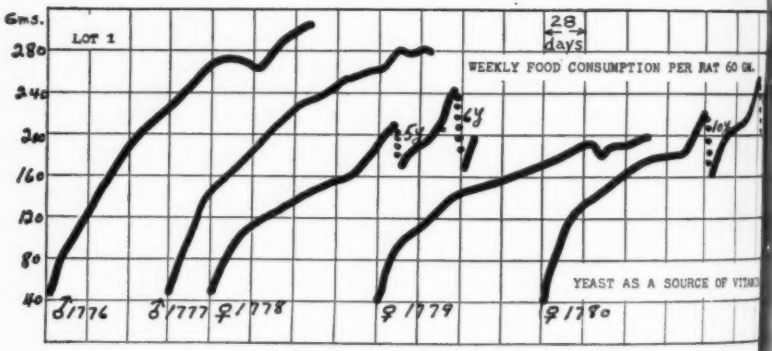


CHART 1.

of these rats (Chart 1) it is seen that this amount of yeast, fed separately, furnished sufficient vitaminB for excellent growth and for reproduction in the case of two females. Neither of these mothers could rear its young.

Lot 2, Chart 2, of this series received the same basal ration as Lot 1. The vitamin B preparation which was fed separately was prepared from the same dry yeast as was used with Lot 1. This preparation corresponded to Fraction II of Osborne and Wakeman's (4) concentrate. It was fed at such a level that the rats received the equivalent of 0.5 gm. of pure dry yeast daily for the

first 8 weeks of the experiment; then the amount was increased to the equivalent of 1 gm. of yeast daily. The rate of growth was not satisfactory (see Chart 2).

Inasmuch as Osborne and Wakeman (4) have secured such excellent growth with as little as 13.2 mg. (the equivalent of 0.2 gm. of yeast) of a like preparation, it might be possible that our failure of growth was due to the fact that our preparation made from a dry yeast was not as efficient as Osborne and Wakeman's which was made from fresh brewers' yeast. However, when we used the same basal ration and the Harris yeast vitamin powder which is prepared essentially by the Osborne and Wakeman method, we obtained no better growth with as much as 240 mg.

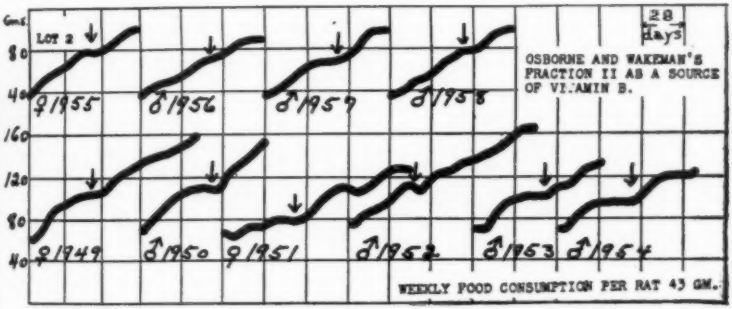
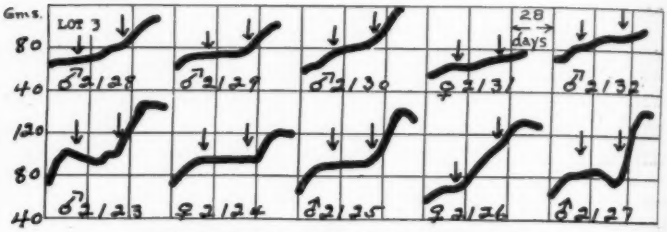


CHART 2.

of the concentrate. It was only when the amount was increased to 320 mg. daily that the rate of growth even temporarily approached the normal (see Lot 3, Chart 3).

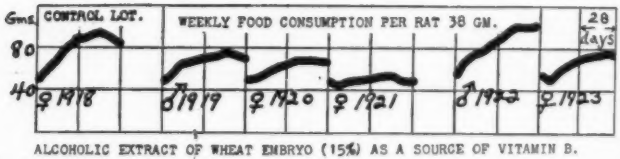
These results are, in general, to be contrasted with those reported in our earlier paper in which we showed that with the procedure then used by us, yeast was not as satisfactory a source of vitamin B as an alcoholic extract of wheat embryo. However, there is this marked difference to be noted between our former and our present laboratory technique; namely, the use of screens to prevent as far as possible the rats' coprophagistic habits. We have shown in another paper (2) that under these conditions young rats will not grow normally when given our laboratory Ration 5 consisting of highly purified casein 18 parts, salts (3) 3.7

parts, agar 2 parts, Crisco 10 parts, butter fat 5 parts, wheat embryo extract equivalent to 15 parts of embryo, and dextrin to make 100 parts. (For growth of rats on this ration, see control lot, Chart 4.) With the exception of greater purity of casein this ration is identical with the one which gave normal growth in



"YEAST VITAMIN-HARRIS POWDER" AS A SOURCE OF VITAMIN B.
WEEKLY FOOD CONSUMPTION PER RAT 38 GM.

CHART 3.



ALCOHOLIC EXTRACT OF WHEAT EMBRYO (15%) AS A SOURCE OF VITAMIN B.

CHART 4.

our former vitamin B experiments. It would therefore appear that yeast not only supplies vitamin B, but some other factor that the alcoholic extract of wheat embryo does not supply. The possibility that the rat is born with such a factor stored in its body cannot be overlooked. Such a supply may be used to meet the needs of growth, be eliminated in the feces as vitamin B[2] has been

[2] Steenbock, Sell, and Nelson (Steenbock, H., Sell, M. T., and Nelson, E. M., *J. Biol. Chem.*, 1923, iv, 399), Dutcher and Francis (Dutcher, R. A., and Francis, E., *Proc. Soc. Exp. Biol. and Med.*, 1924, xxi, 189), Smith, Cowgill, and Croll (Smith, A. H., Cowgill, G. P., and Croll, H. M., *J. Biol. Chem.*, 1925, lxvi, 15), and others have concluded that the difference in growth of rats on screens and on sawdust is to be explained on the basis of a greater vitamin B requirement in the former case. We expect to present our study of the question of the relation of coprophagy to nutritive requirements in rats in greater detail in a subsequent paper.

shown to be eliminated, and then may become a source of supply, provided the rat has access to its feces.

This supposition seems to be well supported by the following experiments in which the yeast and the various yeast preparations used in the above experiments were fed as supplements to a presumably complete ration containing the equivalent of 15 per cent wheat embryo as a source of vitamin B.

Yeast and Yeast Preparations as Supplements to a Presumably Complete Ration.—Lot 4, Chart 5, of this series received as a daily supplement to the "complete" ration 0.2 gm. of starch-free dry yeast (Northwestern Yeast Company). This greatly improved the rate of growth and enabled the rats to reproduce at

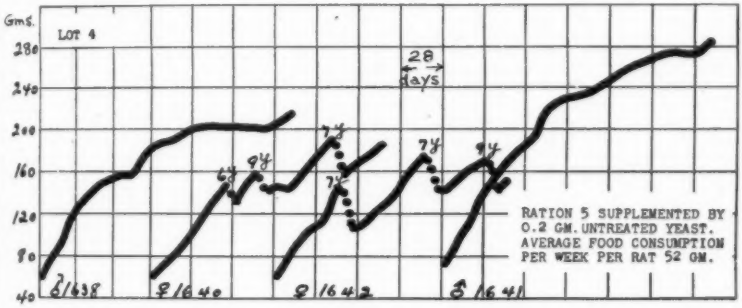


CHART 5.

regular intervals, although the young were not reared. (For comparison, see growth curves of the control lot, Chart 4, and Lot 4, Chart 5.)

A daily supplement of 0.5 gm. of yeast still further augmented the rate of growth, allowing the rats to attain almost the same growth as that of our stock rats on a natural mixed food and milk (see Lot 5, Chart 6). One mother was able to rear a part of one litter with the aid of a daily supplement of milk in addition to the yeast. Even with this the young were extremely slow in growing, and at the end of 6 weeks when weaned, they were very much underweight, approximately 30 to 35 gm., and abnormal in shape and general appearances. When 3 months old, their

weights were 78, 70, and 54 gm. (see Lot 6, Chart 6). For the next 2 months, they made good gains and then began to decline. There was no reproduction in this lot although they were over 6 months old when the experiment was stopped. It is interesting to note that although reproduction was good (see Lot 5) there was no rearing of young, with the exception of the case just cited. This fact does not support Sure's (5) assumption that the factor

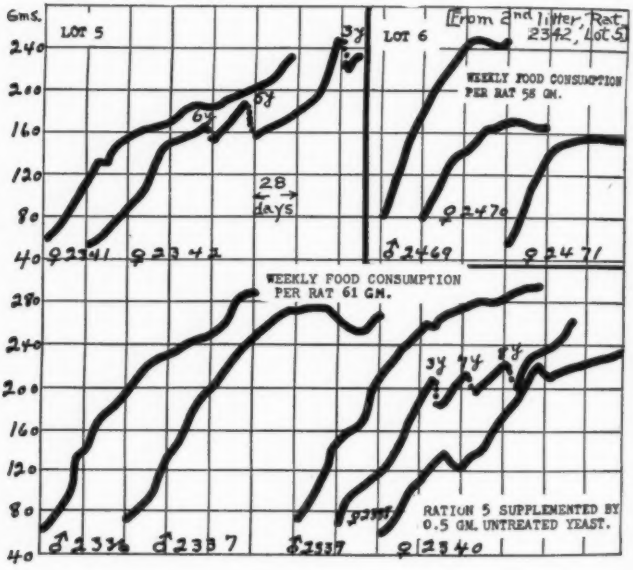

Gms.
LOT 5
LOT 6
From 2nd litter, Rat 2342, Lot 5
WEEKLY FOOD CONSUMPTION PER RAT 58 GM.
28 Days
WEEKLY FOOD CONSUMPTION PER RAT 61 GM.
RATION 5 SUPPLEMENTED BY 0.5 GM. UNTREATED YEAST.

CHART 6.

for reproduction and lactation, if there is one for the latter function, are one and the same. This fact is also out of harmony with the findings of others who have been able to rear several generations on a presumably purified ration and yeast as a source of vitamin B.

We do not believe that the improved growth of Lot 5 is due to increased vitamin B content of the supplemented ration over that of the basal ration. The method employed by us for extracting

vitamin B from the wheat embryo and the amount of this extract which we use in our basal ration assure an ample supply of this vitamin to meet growth requirements (2).

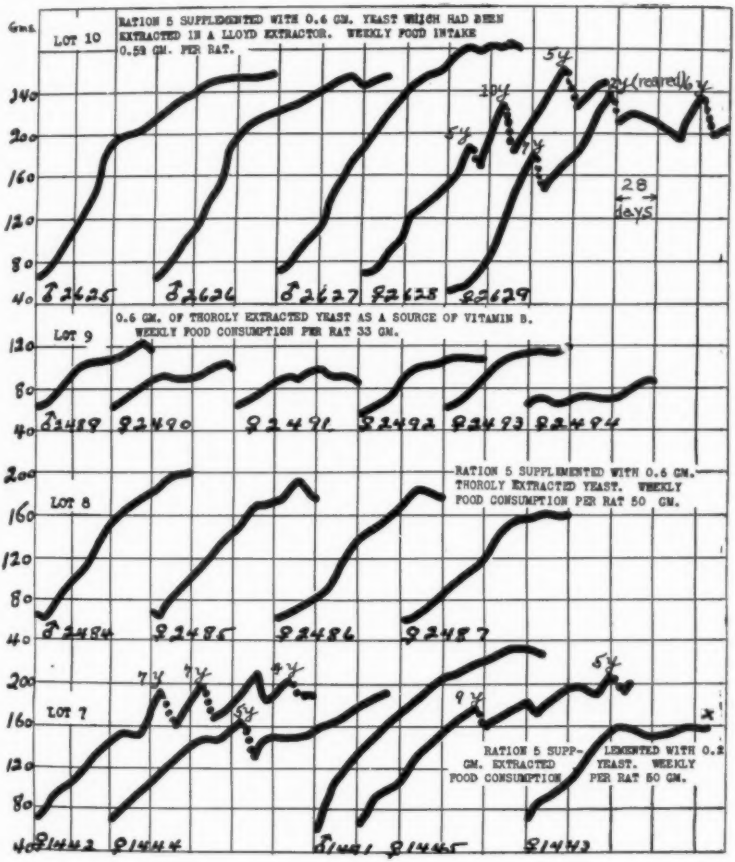

Gms.
LOT 10
RATION 5 SUPPLEMENTED WITH 0.6 GM. YEAST WHICH HAD BEEN EXTRACTED IN A LLOYD EXTRACTOR. WEEKLY FOOD INTAKE 0.52 GM. PER RAT.
♂2625 ♂2626 ♂2627 ♀2628 ♀2629
28 days
LOT 9
0.6 GM. OF THOROLY EXTRACTED YEAST AS A SOURCE OF VITAMIN B. WEEKLY FOOD CONSUMPTION PER RAT 33 GM.
♂2489 ♀2490 ♀2491 ♀2492 ♀2493 ♀2494
LOT 8
RATION 5 SUPPLEMENTED WITH 0.6 GM. THOROLY EXTRACTED YEAST. WEEKLY FOOD CONSUMPTION PER RAT 50 GM.
♂2484 ♀2485 ♀2486 ♀2487
LOT 7
RATION 5 SUPPLEMENTED WITH 0.2 GM. EXTRACTED YEAST. WEEKLY FOOD CONSUMPTION PER RAT 50 GM.
♂14442 ♀14444 ♂14441 ♀14445 ♀14443

CHART 7.

In order to determine definitely that it is not the vitamin B contained in the 0.2 gm. of yeast which renders our Ration 5 growth-

promoting, we attempted to remove vitamin B from yeast by extraction, and fed the residue as a supplement to the ration. Three separate lots of yeast were extracted. Hot 85 to 90 per cent ethyl alcohol was used in each case. The first lot was extracted in a Soxhlet type of extractor which syphoned over about 12 times during the extraction period of 16 hours. The rats (Lot 7, Chart 7) receiving 0.2 gm. daily of this extracted yeast as a supplement to Ration 5 grew at as satisfactory a rate as the rats receiving the unextracted yeast as a supplement (see Lot 4, Chart 5). As there was a possibility that the extraction of vitamin B from the yeast had not been complete, and that the increased amount of this vitamin in the ration was the cause of better growth than we got with the unsupplemented ration, we repeated the experiment using more carefully extracted yeast. The extraction of this yeast was continued until the alcohol which syphoned over was colorless. Twice during the extraction period, the yeast was removed from the thimbles, mixed, and repacked. The alcohol was frequently drawn from the extractor and replaced with fresh alcohol. The extract was dried on dextrin. Both the extract and the extracted yeast were fed separately as supplements to Ration 5, each at levels equivalent to 0.6 gm. of whole yeast daily. From the performance of the rats (Lots 8 and 9, Chart 7), it is readily seen that the extracted residue of the yeast contained something which the extract did not, and that the additional vitamin B contained in the extract did not improve the ration.

The extracted yeast used to supplement the ration of the rats in Lot 10 probably contained even less of the alcohol-soluble vitamin than that used in Lot 8 as this yeast was extracted with hot alcohol in a Lloyd extractor. In this type of extractor, the material to be extracted is surrounded by the hot alcohol which is continually changing. This yeast was also ether-extracted in the same extractor following the alcohol extraction. The equivalent of 0.6 gm. of whole yeast was fed as a supplement. These rats grew at a very satisfactory rate. Reproduction was also good. Only one of the six litters was reared due probably to the fact that there were only two rats in the litter and that the mother rat was kept on a sawdust floor with access to her feces rather than on our usual screen floor. The weight of these two rats when weaned at 30 days of age was 64 gm. each. At the end of 4 weeks on the

mother's ration, one weighed 148 gm. and the other 90 gm. All of the rats receiving this yeast supplement were in excellent condition when the experiment was terminated.

We have further evidence that our ration is not deficient in vitamin B in the performance of the rats in Lots 11 and 12, Chart 8. Each of these groups received Ration 5, and, in addition, the former received 0.25 gm. (the equivalent of 0.5 gm. of starch-free dry yeast) of Osborne and Wakeman's (4) Fraction II, and the latter 0.16 gm. of Yeast Vitamine-Harris Powder during the first period and then 0.24 gm. to the end of the feeding test. These two yeast concentrates which have been recognized as excellent sources of vitamin B did not improve growth as did the addition

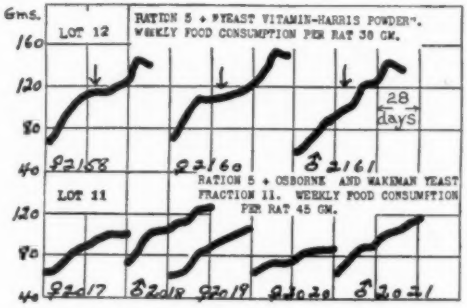


CHART 8.

of 0.2 gm. of starch-free yeast or 0.2 gm. of the alcohol-extracted yeast. In fact, growth was not appreciably better than that of the rats whose sole source of vitamin B was the Harris powder or the Osborne and Wakeman fraction (see Charts 2 and 3). The only conclusion that we can draw from these results is that the vitamin B contained in these two preparations is not needed to supplement the amount already in the ration, and that the Osborne and Wakeman fraction and the Harris powder carry the other factor only in limited amounts if at all.

We regret that we did not have enough of these preparations to continue the feeding experiment for a longer time. But we feel that the results are quite decisive as the general condition of

the rats did not indicate that they could attain a satisfactory rate of growth.

The growth factor other than vitamin B which yeast contains is apparently only very slightly soluble in either water or 85 to 90 per cent alcohol. This is shown not only by the behavior of the rats of Chart 8, but also by those of Chart 9. The yeast residue left after removing the Osborne and Wakeman vitamin concentrates was fed to Lot 13, Chart 9, as the sole source of vitamin B

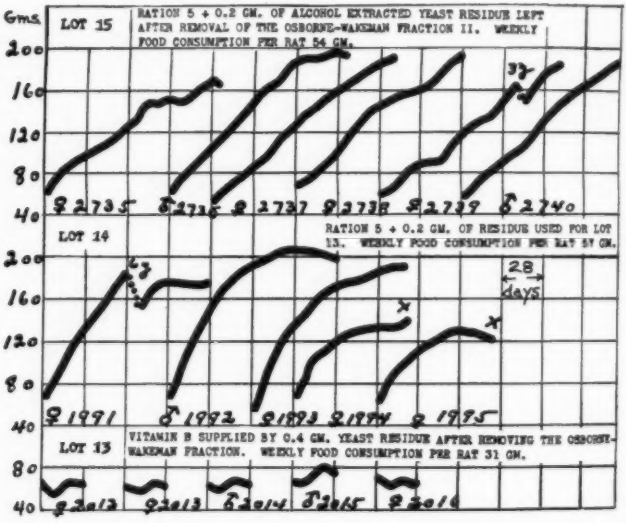


CHART 9.

and to Lot 14 as a supplement to Ration 5. The behavior of Lot 13 shows how completely vitamin B was removed from the yeast. These rats were given 0.4 gm. daily of this yeast residue as a source of vitamin B. The rats of Lot 14 were fed our Ration 5, and in addition, were given 0.2 gm. daily of the yeast residue. In this case, growth was very much better than when our unsupplemented ration was fed, and, in fact, compares very favorably with the growth of the rats shown in Chart 5 where 0.2 gm. of the natural yeast product was fed. The rats of Lot 15 received in

addition to Ration 5, a yeast residue left from the preparation of the Osborne and Wakeman vitamin concentrates. This residue was purified further through extraction with hot 90 per cent alcohol by the method described above. Fresh brewers' yeast was used in this preparation. These rats were given 0.2 gm. of the dry residue daily for the first 6 weeks of the experiment, when the amount was increased to 0.4 gm. This increase did not seem to augment the rate of growth over that of the 0.2 gm. supplement which enabled the rats to grow at a rate that compared favorably with any of the yeast fractions used.

We have also found that Vegex, which is an extract of autolyzed brewers' yeast, and presumably an excellent source of vitamin B, has only very slight stimulating effect when fed as a supplement to our ration. This was reported in a previous paper (6).

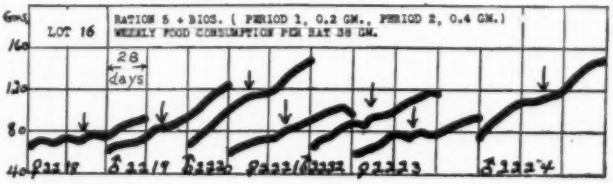


CHART 10.

It seemed worth while to find out whether this factor which apparently is not appreciably extracted from yeast by either water or moderate strength alcohol, was Funk and Dubin's (7) vitamin D or bios, as it is more generally called. We prepared bios according to the methods of Eddy, Kerr, and Williams (8), and fed it as a supplement to Ration 5. The growth curves of Lot 16, Chart 10, show that this preparation did not contain the factor which the untreated yeast and some of our yeast residues do. Funk and Dubin state that young rats given vitamins B and "D" from the start, together with the last filtrate from the preparation of these two vitamins, which contains neither B nor "D," showed a normal behavior both in regard to growth and appearance. It is possible that this filtrate contains the factor which supplements our ration.

After the foregoing experiments were completed, Goldberger,

Wheeler, Lillie, and Rogers (9) published their paper on the relation of factor P-P of pellagra to vitamin B in yeast. This stimulated a further study by us of the factor other than vitamin B which we had found to be present in yeast. At the outset, it seemed improbable that we were dealing with the P-P factor

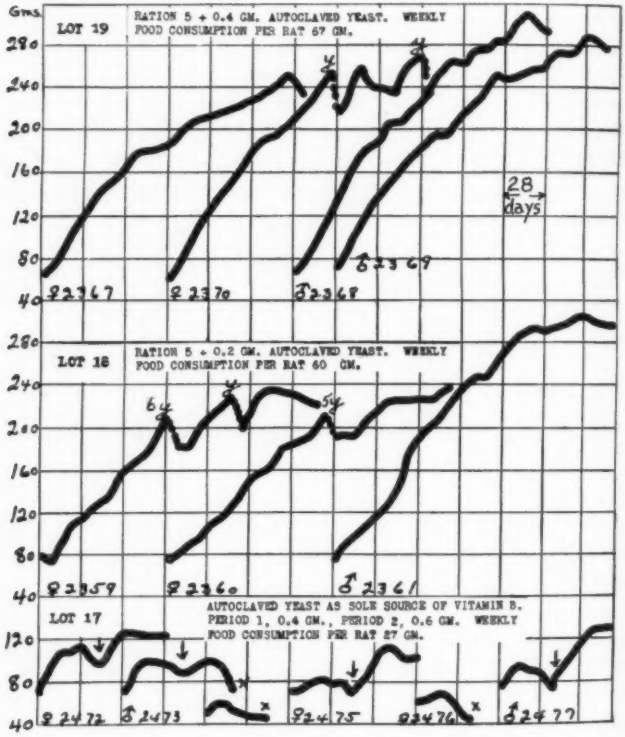

Gms.
LOT 19
RATION 5 + 0.4 GM. AUTOCLAVED YEAST. WEEKLY FOOD CONSUMPTION PER RAT 57 GM.
28 days
♀ 23 67
♀ 23 70
♂ 23 68
♂ 23 69
LOT 18
RATION 5 + 0.2 GM. AUTOCLAVED YEAST. WEEKLY FOOD CONSUMPTION PER RAT 60 GM.
♀ 23 59
♀ 23 60
♂ 23 61
LOT 17
AUTOCLAVED YEAST AS SOLE SOURCE OF VITAMIN B. PERIOD 1, 0.4 GM., PERIOD 2, 0.6 GM. WEEKLY FOOD CONSUMPTION PER RAT 57 GM.
♀ 24 72
♂ 24 73
♀ 24 75
♀ 24 76
♂ 24 77

CHART 11.

because Goldberger and coworkers found the factor present in Yeast Vitamine-Harris Powder, an acidulated watery extract of yeast. We had found that the Yeast Vitamine-Harris Powder stimulated growth only very slightly when added in liberal amounts to our complete ration, and also, that acidulated water did not

extract the necessary growth factor from yeast. Smith and Hendrick (10), however, had reported that autoclaved yeast contains an essential growth factor. Goldberger and his associates likewise had found their P-P factor to be thermostable, to be distinct from the antineuritic vitamin, and, although not growth-producing by itself, to be essential in combination with the antineuritic factor for growth in rats. It, therefore, seemed of interest to determine the effect of autoclaving yeast on its growth-promoting power in our diets.

We autoclaved dry starch-free yeast (Northwestern Yeast Company) in 250 to 300 gm. quantities in quart Mason jars with loosely fitting caps for 2½ hours under 15 pounds pressure. When taken from the jar, the yeast was somewhat plastic in character, though not in a moist or gummy state. It was quickly dried at a low temperature and made into pellets weighing 0.2 gm. We fed this yeast both as the sole source of vitamin B and as a supplement to our Ration 5, containing wheat embryo extract. When used as the sole source of vitamin B, 0.4 gm. daily did not prevent rapid decline after the 5th week; an increase to 0.6 gm. daily was also insufficient to sustain growth for longer than 3 weeks (see Lot 17, Chart 11).[3] The antineuritic property of the yeast had also been destroyed by the heating as 0.6 gm. daily did not prevent pigeons from getting polyneuritis when fed a polished rice diet. However, when used as a supplement to Ration 5, very satisfactory growth was attained when 0.2 gm. per day was fed. When 0.4 gm. per day was fed the growth was somewhat better. The condition of both lots of rats (Lots 18 and 19, Chart 11) was exceptionally good throughout the experiment. It is evident that autoclaved yeast retains the growth factor necessary for our Ration 5, just as it retains the factor required for Smith and Hendrick's diets and the P-P factor in the experiments of Goldberger and associates.

DISCUSSION.

We have described in this paper feeding experiments in which we have used yeast and yeast preparations both as sources of vitamin B and as supplements to a presumably complete ration.

[3] In a later experiment it was found that even 1 gm. per day of the autoclaved yeast did not promote growth.

The outstanding result of the experiments in which these materials were used as a source of vitamin B is the failure of the rats to grow satisfactorily when any of these materials except the yeast in its natural state was used. In other words, neither of the yeast preparations which are commonly and evidently successfully used in other laboratories promotes even a fair rate of growth under our method of experimentation.

The only explanation we can offer for such a situation is that there is another growth factor which has not yet been recognized. This factor may either be carried by some of the constituents of the ordinary purified diet, possibly the casein, or the young rat may have this factor stored in its body and eliminate it in the feces, thereby providing a source of supply unless coprophagy is prevented.[4] Thus the factor may be available to the rat on a synthetic ration unless extreme caution is taken to exclude it.

The growth of the rats receiving the Osborne and Wakeman concentrate or the Yeast Vitamine-Harris Powder though very unsatisfactory, was better than that of most of the rats which have received our usual Ration 5 containing the equivalent of 15 per cent wheat embryo extract as a source of vitamin B (2). The amounts of these yeast preparations given the rats should have been ample to meet their vitamin B requirements; at least they were much in excess of the amounts prescribed by others. For example, Osborne and Mendel (11) state that 40 mg. of this concentrate are sufficient for growth to almost 200 gm. in body weight. It is apparent that extracts made from either yeast or wheat embryo are not entirely devoid of the factor present in the whole yeast. Unlike vitamin B, the factor seems to be much less soluble in alcohol or water, or at least much less readily extracted from yeast by these solvents. This would account for the presence of only small amounts in preparations which are made from watery or alcoholic extracts of yeast. When these preparations were used as supplements to our Ration 5, there was some improvement in the rate of growth of rats over that of rats getting the unsupplemented ration, but there was no appreciable difference

[4] We find that the most satisfactory way of minimizing coprophagy is by the use of a screen floor of sufficiently large mesh, *i.e.* at least 3 mesh per inch, to allow the feces to drop through, and elevated high enough above the floor of the cage to prevent the rat reaching the feces.

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in their rate of growth and that of the rats which had received the same or larger portions of these preparations as the sole source of vitamin B. Such results strengthen our belief that our complete ration contains an ample amount of vitamin B in the form of wheat embryo extract, but that it lacks some other factor which yeast supplies.

However, the most convincing proof that yeast contains something other than vitamin B, which our complete ration lacks, is found in the results of the supplementary feeding of the autoclaved yeast, the yeast which had been thoroughly extracted with alcohol and the residue left after removing the vitamin fractions in Osborne and Wakeman's concentrates. The vitamin B had been removed from the two latter preparations. Neither of these preparations would promote growth when used by itself as source of vitamin B, yet, when they were used as supplements to our Ration 5, they produced very satisfactory growth. In the autoclaved yeast, both antineuritic and the growth-promoting vitamin B had been destroyed, yet small amounts gave very satisfactory growth when used to supplement our Ration 5.

SUMMARY AND CONCLUSION.

1. We have shown that a highly purified ration presumably complete except for vitamin B is made adequate for the growth of rats when each animal receives daily a 0.6 gm. pellet of dry starch-free yeast.

2. We have shown that the same basal ration is not made adequate for growth when the source of vitamin B is either the Osborne and Wakeman concentrate from pure dry yeast, or the residue left from the preparation of this concentrate, or a yeast concentrate supplied by the Harris Laboratories, or dry yeast which has been autoclaved 2½ hours at 15 pounds pressure.

3. We have shown that 0.2 gm. daily doses of either dry starch-free yeast, or the same kind of yeast which has been very thoroughly extracted with hot 85 to 90 per cent alcohol, or the residue left from the same kind of yeast after removal of the Osborne and Wakeman vitamin fractions, or yeast that is autoclaved to destroy its recognized growth-promoting and antineuritic properties, will supplement a non-growth-promoting ration already containing an adequate amount of vitamin B in the form of an extract of

wheat embryo so that average growth and reproduction are attained.

4. We have shown that neither the Osborne and Wakeman vitamin B Fraction II prepared from dried yeast, nor the Harris Laboratory preparation from brewers' yeast, nor an alcoholic extract of dried yeast carries adequate amounts of the same growth factor that is present in (1) the alcohol-extracted yeast, (2) the residue left after removing the Osborne and Wakeman concentrate, or (3) autoclaved yeast.

5. We have been unable to show that the growth factor is bios.

6. We have shown that the factor is only slightly extracted from yeast by alcohol or acidulated water and is stable to autoclaving at 15 pounds pressure for 2½ hours.

7. We conclude, therefore, that yeast contains some factor other than vitamin B or the antineuritic factor, and that this factor is necessary for satisfactory growth. The evidence so far collected indicates that the factor is in some respects similar to, but not identical with the yeast growth factor of Smith and Hendrick(10) and the P-P factor of Goldberger and associates (9).

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THE FUNDAMENTAL FOOD REQUIREMENTS FOR THE GROWTH OF THE RAT.

IV. COPROPHAGY AS A FACTOR IN THE NUTRITION OF THE RAT.*

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The habit of the rat to consume the feces when confined to a cage is commonly observed in nutrition laboratories which use this animal for experimental work. The effect of this depraved appetite on the general state of nutrition of this species, when on natural and artificial diets, or upon the outcome of various nutrition experiments has not been entirely worked out. The problems introduced into nutrition studies by the prevention of coprophagy are probably not as simple as recent work has tended to indicate.

The present paper gives a résumé of our experience in studying the effect of feces eating in rat nutrition studies with synthetic diets, and suggests the belief in the complexity rather than the simplicity of the problem. If the feces from such diets consisted only of indigestible food materials, coprophagy would not be a problem in nutrition. However, when we consider that the feces are made up not only of products of exogenous metabolism but also of endogenous metabolism, epithelial cells from the intestinal walls, the products of organisms as well as the organisms themselves, and undigested food material, we are confronted by a serious problem that cannot be disregarded.

In 1911, when the biological method of investigation was in its early stage of development, Osborne and Mendel (1) noted the probable importance of maintaining the normal bacterial flora of the alimentary tract. They believed that the water-free, fat-rich food characteristic of their ex-

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perimental diets was not a favorable medium for the development of a normal flora, and that this was a contributory cause to the ultimate nutritional failure of their rats. They found that small additions (0.5 gm. twice a week) of the air-dried feces of rats on an ordinary mixed diet would, in nearly every instance, stop decline in their experimental rats, and that even sterilized feces, *i.e.* feces heated three times with steam, were beneficial though much less potent. As this work of Osborne and Mendel's was done before vitamins were discovered, the beneficial effect of coprophagy was not explained as an effect of addition of any factor to the diet, but rather to the implanting of a normal bacterial flora in the intestine. Rettger and Horton (2) used this idea of Osborne and Mendel's as the basis for an extensive investigation. They came to the conclusion that differences in the composition of the diet could bring about marked changes in the intestinal flora; that on their simplified diets, that is definite portions of pure protein, starch, lard, and protein-free milk, the number of types of bacteria became greatly reduced, but that no definite relationship could be established between the well being of the animal and its intestinal flora. It is, therefore, necessary to attribute the beneficial effects found by Osborne and Mendel to something carried by the feces.

The relation of coprophagy to nutrition has recently been given a new emphasis through the work of Steenbock and his associates (3). They believe that coprophagy in rats reduces their vitamin B requirement by at least one-half. These investigations show that a ration, which had always given fairly satisfactory growth when the rats were on sawdust, could only maintain a rat after 3 to 4 weeks when screens were used to prevent coprophagy. This same ration could be made satisfactory for growth on screens when the source of vitamin B (wheat embryo) was doubled. They also show that from 2 to 3 times as much yeast is required in the ration as a source of vitamin B when the rats are left on screens as when they are kept on shavings. Dutcher and Francis (4) believe that the rat can store sufficient vitamin B in its tissues to maintain a fair degree of growth for 4 to 5 months on a vitamin B-free diet provided coprophagy is allowed from the beginning of the experimental period. On the other hand, such a store of vitamin is depleted within 2 weeks if the rats are immediately placed on screens, and after this period, coprophagy no longer stimulates growth. Smith, Cowgill, and Croll (5) have collected data, under carefully controlled conditions, which show that the average requirement of vitamin B for normal growth to 120 gm. of body weight is 33 mg. of vitamin powder (Harris) when screens are used to prevent coprophagy, while only 20 mg. of the same powder suffice when no screens are used.

Opposed to this view that coprophagy may add measurable amounts of vitamin B to the ration, is that of McCollum and his coworkers (6), who affirm that with the laboratory technique employed by them (*i.e.* the rats are kept on sawdust and cages are cleaned once in 2 weeks) any vitamin content of the feces derived from vitamin-containing food is very small after the first 3 to 6 days, and practically non-existent after the second cleaning of the cage. This does not agree with the findings of Dutcher

and Francis, who showed that coprophagy must be prevented from the beginning of the experiment in order not to delay the results of a lack of vitamin B in the ration for 4 or 5 months.

Heller, McElroy, and Garlock (7) agree with other investigators that the growing period is extended on a vitamin B-deficient diet when the feces are consumed by the rat, and have definitely shown that vitamin B is synthesized by the spore-bearing organisms found in the feces. They believe, however, that the final results of the biological test for vitamin B are not different under the two conditions, that is with screens or without them, because the spore-bearing organisms disappear from the feces by the end of the 3rd week on a vitamin B-deficient diet.

EXPERIMENTAL.

In April, 1923, we began the following experiments in an attempt to find out whether the failure of our rats to grow normally on our Ration 5 was due to the fact that the rats did not have access to their feces and were thus deprived of a factor present in the feces and necessary for growth on highly purified diets. Our general laboratory technique and preparation of foods have already been described in detail (8). The colony plan of housing was used throughout the experiments; the rats were weighed weekly and records of food intake and feces consumed were kept for each group. When coprophagy was to be prevented, wire screens (3 meshes to the inch), raised 1 inch above the floor of the cage, were used; otherwise the rats were on sawdust which was changed once a week when the cages were cleaned. The ration that was used throughout the experiment was our highly purified Ration 5 consisting of purified casein 18, salts (McCollum's 185 (9)) 3.7, agar 2, butter fat 5, salad oil (Wesson) 10, the alcoholic extract of ether-extracted wheat embryo equivalent to 15 per cent of the ether-extracted embryo, and dextrin to make 100 parts.

The effect of coprophagy on growth is shown in the growth curves of Lots 1, 2, and 3, Chart 1. In these groups, the rats were left on a screen flooring from the beginning of the experiment to the end of the 7th week in Lots 1 and 2, and to the end of the 6th week in Lot 3, during which time they either barely maintained themselves or actually declined in weight. At this time, the screen floor was removed and growth was immediately resumed, and although it was not commensurate with the normal growth of our stock rat, it was distinctly better than it had been. As the cages had been cleaned and the sawdust renewed weekly from the be-

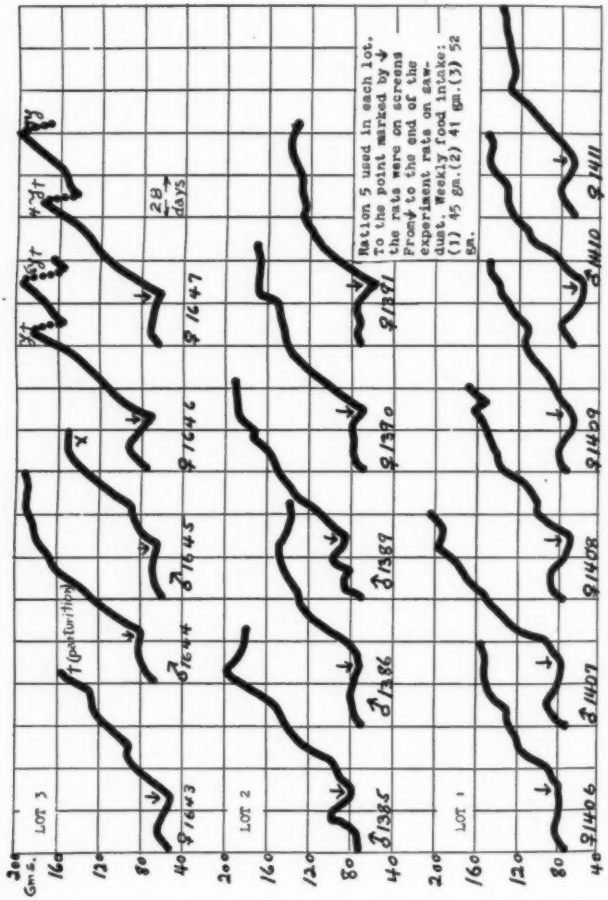
LOT 3
LOT 2
LOT 1
Gms.
1st (parturition)
28 days
Ration 5 used in each lot. To the point marked by ↓ the rats were on screens From ↓ to the end of the experiment rats on sawdust. Weekly food intake: (1) 45 gm. (2) 41 gm. (3) 52 gm.

CHART 1.

ginning of the experiment, the feces which the rats consumed could not have carried any material from the mixed food which the young rats had been fed before being placed on the experimental ration. However, it is possible to postulate that some factor necessary to the rat's well being is synthesized by the bacteria inhabiting its intestinal tract, and is excreted into the fecal material and thus lost to the rat unless coprophagy is allowed. Whatever the material may be, it undoubtedly causes the rat to recover from its precarious condition and to grow, although at a subnormal rate. It does not seem probable that the feces can add anything to the protein, inorganic salts, or energy content of the ration. Osborne and Mendel (10) have shown that 18 per cent of casein is ample to take care of the protein requirements of the rat; McCollum has suc-

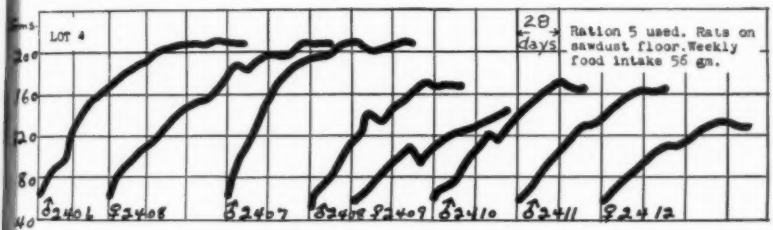

CHART 2.

cessfully used Salt Mixture 185 for many years; the weekly food intake of all the rats was more than enough to supply the 40 large calories per 100 gm. of weight that Drummond's experiments (11) suggest as necessary for growth; and in a recent paper, we (12) have shown that the effect of vitamin B supplied by the alcoholic extract of 15 gm. of ether-extracted wheat embryo per 100 gm. of ration is not intensified by liberal additions of other vitamin B preparations, such as Yeast Vitamine-Harris Powder or a similar preparation made by us from yeast by Osborne and Wakeman's method (13). Increasing the vitamin A and vitamin D content of this ration has never enhanced its value. It would seem, therefore, that the value of the feces lies in some other constituent.

Apparently it does not make any difference in the results of the experiment whether coprophagy is permitted from the beginning

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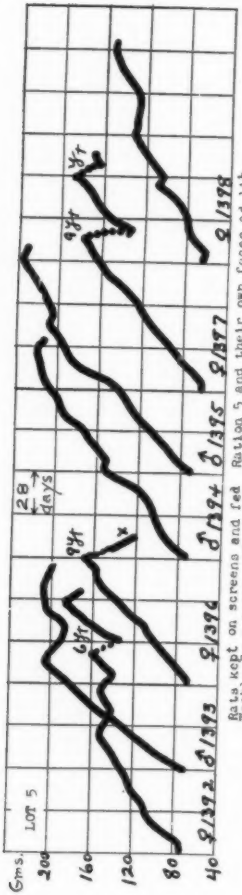
Gms.
LOT 5
200
160
120
80
40
28 days
♀/372
♂/373
♀/376
♂/374
♂/375
♀/377
♀/378
Rats kept on screens and fed Ration 5 and their own feces ad lib.
Weekly food intake 46 gm. per rat.

CHART 3.

of the feeding period as in Lot 4, Chart 2, or only after several weeks have passed as in Lots 1 to 3. The final weight attained by the rats and their rate of growth during the feces-consuming period, is the same in each experimental group. This is in harmony with our assumption that the substance in the feces which stimulates growth is not carried over from a previous diet, but is either a dietary constituent, whose conservation after digestion and metabolism depends upon coprophagy in the rat, or is a substance synthesized in the digestive tract of this species.

In Lot 5, Chart 3, a slightly different procedure was followed. The rats were on screens for the 1st week of the experiment and did not have access to any feces. From the end of the 1st week to the end of the experimental period, each week when the cage was cleaned the feces were collected, freed of sawdust, dried, and fed *ad libitum*. The daily consumption of feces averaged 2 gm. per rat. This method of feeding the feces was used to determine whether the beneficial effect of removing the screen was due to the consumption of feces or sawdust. It was evidently not due to the sawdust, but to the feces.

The results of these experiments indicated that there is something in the feces which aids greatly in the growth and well being of the rats fed our synthetic Ration 5. However, whatever the substance is, it seems to be present in limited amounts so that satisfactory growth cannot be attained even though the rats can consume the feces *ad libitum*. This is seen by observing the growth of the rats in Lot 5 (Chart 3). Although growth was much better than when the rats are deprived of access to the feces, still it cannot be regarded as optimum.

The above results suggested that if such a factor is present in the feces of rats on a purified diet in small though inadequate amounts, it might be present in greater concentration in the feces of rats living on a natural mixed food. We, therefore, fed the feces of our stock rats which are given a mixed ration of natural foods and milk. The growth of the first generation of rats receiving these feces, Lot 6, Chart 4, was not significantly better than that of the rats which had received their own feces, although reproduction and the rearing of young was greatly improved. The comparison should be made between the growth curves of Lot 5 and Lot 6, as the same method of feeding was used in each group. The

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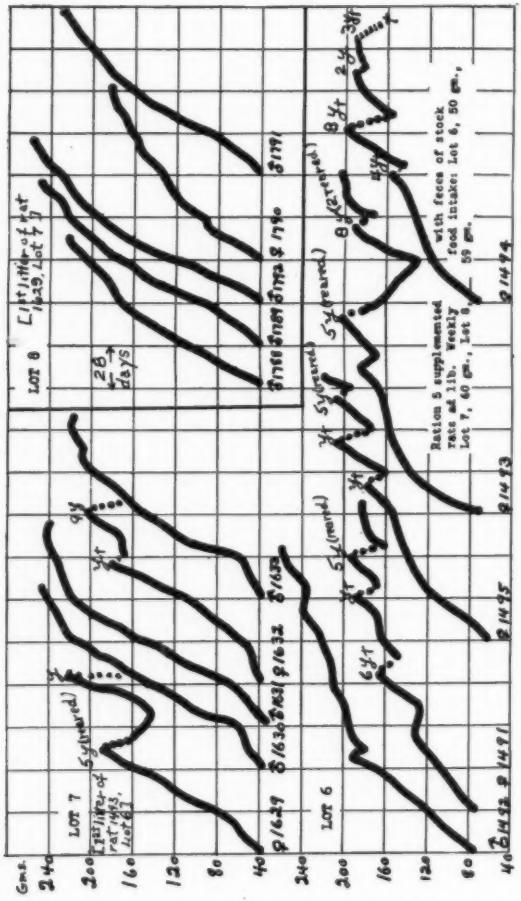
Gms.
240
200
160
120
80
40
LOT 7
LOT 6
LOT 8
28 days
Ration 5 supplemented rats at 11h. Weekly
with feces of stock
food intake: Lot 6, 50 gm.,
59 gm.

CHART 4.

much better growth in the first and second generation young, Lots 7 and 8, Chart 4, than in the parent stock may be due to the accumulative effect of the better diet. Sherman has recently shown that improvement in health and vigor due to improvement in diet is even more noticeable in the second generation than in the first (14).

The fact that the rats in the two feces-fed groups grew at apparently the same rate strengthens the supposition that the feces do not supplement the ration by adding one of the recognized food

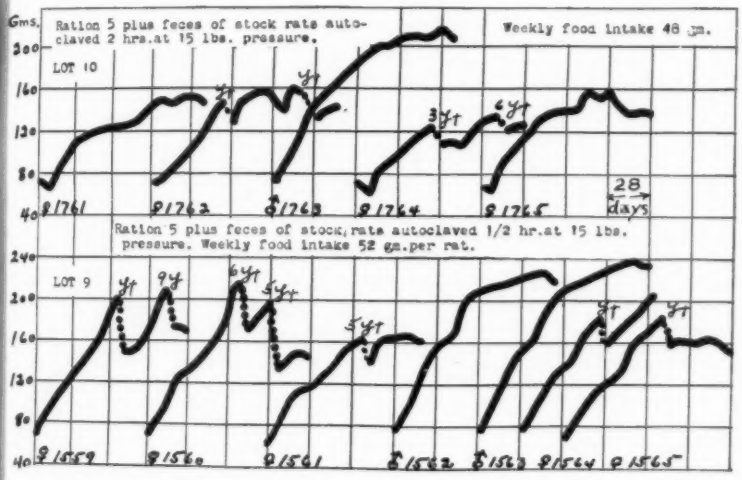

CHART 5.

factors to the deficient ration. It is reasonable to suppose that the feces of the stock rats contain more undigested material than is present in the feces of rats fed a purified synthetic diet. It, therefore, seems probable that the similarity between the effect of the two kinds of feces was due to some substance which they possessed in common, but which was not a known substance resulting from inadequate digestion.

We have given some consideration to the biological character of this factor. From the evidence we have accumulated, it seems very probable that the substance belongs in the group of vitamins.

XUM

It is growth-stimulating, is injured by prolonged heating at high temperature, and is soluble in alcohol. Moderate heat, that is heating the feces for ½ hour at 15 pounds pressure, does not injure their growth-stimulating properties. The rats of Lot 9, Chart 5, received 2 gm. daily of feces thus heated. The feces came from the stock rats. However, heating for 2 hours caused considerable deterioration (see Lot 10, Chart 5).

The solubility of this factor in alcohol is shown by the following experiment. Each of the rats of Lot 11, Chart 6, was given as a supplement to its daily ration, feces extract equivalent to 2 gm. of feces. This material was prepared by extracting the feces of

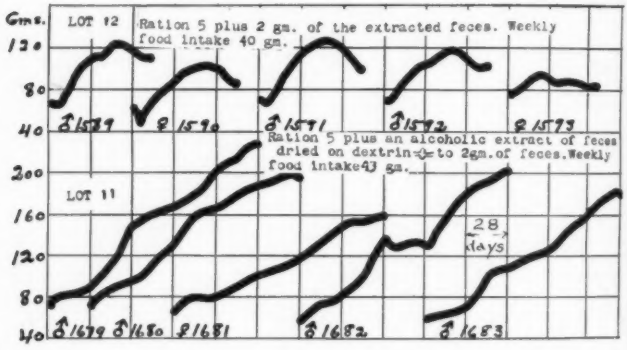

CHART 6.

our stock animals with hot 90 per cent alcohol for 24 to 32 hours in a Soxhlet type of extractor. The extract was dried on dextrin. The feces which had been extracted were fed at a level of 2 gm. per day to Lot 12, Chart 6. Apparently the alcohol entirely removed the stimulating factor from the feces as the growth of these rats was no better than we have often had when our Ration 5 was the sole source of food.

Although this factor is removed from the feces with 90 per cent alcohol, we do not believe that it is vitamin B because we have abundant evidence that our Ration 5 carries a sufficient amount of growth-promoting vitamin B. In an earlier paper (12) we showed that our Ration 5, which carries the alcoholic extract of 15 per

cent of wheat embryo, does not give satisfactory growth when the vitamin B content is increased by a daily supplementary feeding of 0.24 gm. of the Yeast Vitamine-Harris Powder or by Osborne and Wakeman's Fraction II, equivalent of 0.5 gm. of starch-free yeast. Each of these preparations used as the sole source of vitamin B has given satisfactory growth in other laboratories when fed at much lower levels. If our ration was deficient in growth-promoting vitamin B, these additions would certainly be ample to meet the requirements for this vitamin. At the present time, we are not prepared to discuss the possible relationship between the factor found in feces and the one that is found in yeast after the removal of the alcohol-soluble vitamin. The evidence so far obtained does not point to their identity. It is possible, however, that the difference in solubility of these two factors is due to a

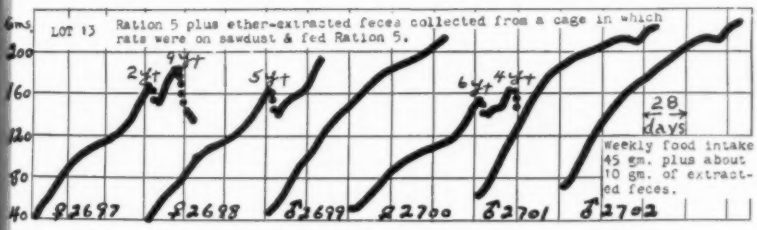
Gms.
200
160
120
80
40
LOT 13
Ration 5 plus ether-extracted feces collected from a cage in which rats were on sawdust & fed Ration 5.
28 days
Weekly food intake 45 gm. plus about 10 gm. of extracted feces.

CHART 7.

difference in the way in which they are combined in the two very different products, feces and yeast. This might explain also the apparent difference in their heat stability.

The growth-stimulating substance found in the feces is not removed by ether extraction. The feces collected from the cage of rats kept directly on a sawdust-covered floor and fed our Ration 5 were extracted with ether in a percolator type of extractor. The rats in Lot 13, Chart 7, were fed, in addition to their usual ration, approximately 1.5 gm. of the ether-extracted feces. There is no difference in the rate of growth of these rats and those of Lot 5, which were given unextracted feces.

It is evident that coprophagy greatly improved growth in rats fed our highly purified Ration 5. However, in no instance is the growth as good as that of our stock rat fed a mixed diet and milk.

Gms.
LOT 15
Ration the same as fed to Lot 14. Rats on screens. Weekly food intake 55 gm.
♂ 1232
♀ 1233
♀ 1234
♂ 1235
♂ 1236
28 days
LOT 14
Ration made to resemble McCollum's complete ration as nearly as possible. Rats on sawdust. Weekly food intake 80 gm.
♀ 1225
♀ 1226
♀ 1227
♂ 1228
♂ 1229

CHART 8.

It cannot even be said to be satisfactory for rats on an experimental ration.

As other investigators have given the impression that their complete synthetic rations were satisfactory from at least a growth standpoint, we decided to attempt a repetition of some reported work. McCollum (6) has definitely stated that he prefers a sawdust floor in his experimental cages, and since the proportions of our Ration 5 were based on his complete ration (15), we wished to find out, if possible, whether his ration would give the same results when coprophagy was prevented as when it is permitted, and also, whether the results obtained from preventing coprophagy would resemble the results which we obtain with our Ration 5

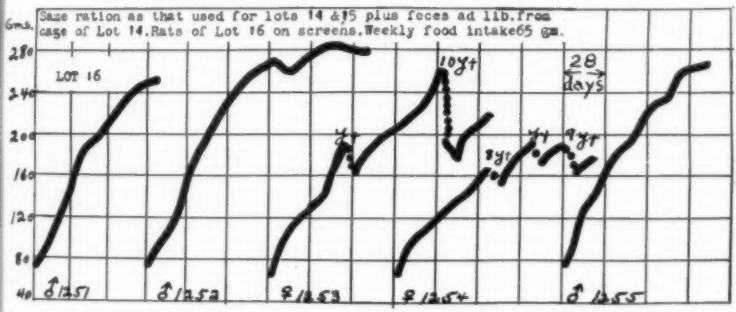

CHART 9.

under like conditions. We, therefore, prepared the food materials of his synthetic ration using the methods for preparation as given by him and his associates, fully appreciating that it is almost impossible for two laboratories to manipulate material in exactly the same way.

Lots 14 and 15, Chart 8, were fed this ration, the former lot being kept on sawdust and the latter on a screen floor to prevent coprophagy. The rats of Lot 16, Chart 9, were started on the experimental ration 1 week later than those in Lot 14, and were prevented access to their feces and the sawdust by a screen flooring. These rats were given *ad libitum* the feces collected weekly from the cage of Lot 14.

The growth curves of these rats are very significant when com-

XUM

pared to those of Lots 1, 2, and 3, Chart 1. The rats of Lots 1, 2, and 3 made no gains in weight when coprophagy was prevented, while those of Lot 15, kept under the same conditions, made fairly satisfactory growth from the beginning of the experiment. The food materials of which the two rations were made were the same with the exception of the casein. The methods used in the preparation of the two caseins are so unlike that the assumption that the difference in the two rations is in the protein is not unwarranted. The casein used in the ration of the rats of Lots 14, 15, and 16, was prepared by the method used by McCollum (16), while that used in the ration of the rats in Lots 1, 2, and 3, was prepared by the method used in this laboratory (8). The important difference in these two methods is that in the former method, purification involves the washing of commercial casein with acidulated water, while the latter method involves the precipitation of casein from fresh skim milk, its reprecipitation from dilute ammonia water, followed by complete extraction with water, alcohol, and ether, without previous drying.

The contrast in the rate of growth of Lot 14 on sawdust and Lot 15 on screens is not at all marked, while the difference in the rate of growth of Lots 1, 2, and 3 when on screens and on sawdust is very marked. This also would seem to indicate that the ration itself of Lots 14 and 15 carried something which the ration of Lots 1, 2, and 3 did not, and which was supplied, in part, by the feces. The rats of Lot 16 fed the feces from Lot 14, made somewhat better growth than the rats in the other two lots (Nos. 14 and 15), which was probably due, in part, to the fact that their food intake was considerably greater and, in part, to the fact that they ate more feces than the rats kept on sawdust.

Our results thus far reported (8, 12, 17) point strongly to the fact that some factor or factors are necessary to make a complete growing ration out of our Ration 5 consisting of an adequate protein, salt mixture, vitamins A and B, and energy-yielding material. We have previously shown (12) that something is supplied by yeast which makes this ration a much better one for growth, and we have shown in this paper that feces, produced both on a normal mixed ration and on a purified ration, aid in promoting better growth. In unpublished work, we have also had further evidence that the feces contain something which makes for normal growth

and reproduction of rats on natural diets. On comparing a group of rats fed on our stock ration but kept under the same living conditions as are our experimental rats, that is on screens, with stock rats on sawdust, we found that the seventh generation young of rats on screens ceased to grow to the usual adult size of our stock rat, to reproduce normally, and to rear their young. We feel certain that this is due, in part at least, to the fact that coprophagy is prevented by the screen flooring in the cage.

SUMMARY.

1. Decline in weight in our experimental rats fed our highly purified presumably complete ration was stopped, and fair gains in weight made by allowing the rats to consume their feces.

2. The feces of rats on a mixed diet were no more potent in stimulating growth than the feces of rats on a highly purified synthetic diet.

3. The factor present in feces, which is growth-stimulating, was found to be injured by prolonged heating, soluble in alcohol, and insoluble in ether.

4. The employment in the basal diet of commercial casein purified merely by leaching with acid water was found to overcome the necessity of coprophagy made evident by the use of highly purified casein.

CONCLUSION.

We conclude from the evidence presented that the feces act beneficially because of some unrecognized factor which they carry.

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BLOOD CONCENTRATION IN MARINE FISHES.

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INTRODUCTION.

The influence of asphyxiation on the concentration of certain blood constituents was discussed by the writer in a previous paper (5). It is apparent that asphyxiation produces a definite increase in concentration of hemoglobin, phosphorus, iron, non-protein nitrogen, total nitrogen, number of red blood cells, and percentage of dry matter. It was also shown that the chloride concentration was maintained nearly constant during asphyxiation. It was concluded that in asphyxiation water was released from the blood, and hence there is a decrease in the blood volume with an increase in the various blood constituents.

The results reported in this paper represent a continuation of the work on asphyxiation. Blood analyses were restricted to the following constituents: iron, hemoglobin, red blood cells, total nitrogen, phosphorus, and oxygen capacity. Analyses were also made of iron found in the spleen and on the weight of the spleen before and after asphyxiation of the fish.

It was noticed that the spleen changed in size during asphyxiation of the puffer fish in some preliminary experiments made in collaboration with Drs. Hisaw and Gray at Woods Hole during the summer of 1926. These observations induced the writer to carry on the work reported in this paper in the laboratories of the United States Bureau of Fisheries at Woods Hole during the summer of 1927. The author is indebted to the Bureau for assistance in the investigation, and to Mr. R. L. Pearse for help in analysis.

Methods.

Fishes used in this experiment were the menhaden, *Brevoortia tyrannus* Latrobe, and the puffer fish, *Spheroides maculatus* Block and Schneider. They were caught in traps and were brought to the laboratory in "live cars." They were left for 2 days in a large floating live car in order to obtain a minimum variation in the blood constituents.

To obtain different degrees of asphyxiation, fishes were placed separately in museum jars containing approximately 10 liters of water. The jars were immersed in a small hatchery box, one jar in each box. Water was kept running through the boxes. The jars were sealed with glass stoppers. Thus the temperature was kept constant and the exact degree of asphyxiation determined for each fish. The Winkler method for the determination of dissolved oxygen as modified by Birge and Juday (2) was used. Menhaden were removed from the jars at 10 minute intervals. The puffers were removed at 30 minute intervals. Menhaden were bled by cutting off the tail and the blood was caught in an Erlenmeyer flask containing lithium oxalate as an anticoagulant. Puffers were bled from the heart with a hypodermic syringe. The temperature of the asphyxiating fish was maintained between 19–20°.

In the analyses of the blood the following methods were employed. Hemoglobin was determined by the Newcomer method. A biological colorimeter fitted with a Newcomer standard for acid hematin was used. The red blood corpuscles were enumerated by the standard clinical method. A counting slide with a Neubauer ruling was used. Total nitrogen was determined as directed by Folin's manual (3). About 2 cc.of whole blood were weighed and dried at 95° until the weight became constant. The ratio of the dry weight to the initial weight was thus determined. Iron was determined by the Wong method. It was found that if the sulfuric acid was diluted to about two-thirds strength instead of the concentration given by Wong (6) the color would not fade as rapidly and that more accurate readings could be made.

The spleens were removed before bleeding the fish after a double ligation of the blood vessels supplying the spleen. Thus no blood was lost from either the spleen or the rest of the body. The spleens were then placed separately in dry weighing bottles and weighed

on an analytical balance. They were then transferred to a Pyrex test-tube and iron determinations made by the Wong method. After the first few attempts, the spleens were extirpated easily within a few seconds.

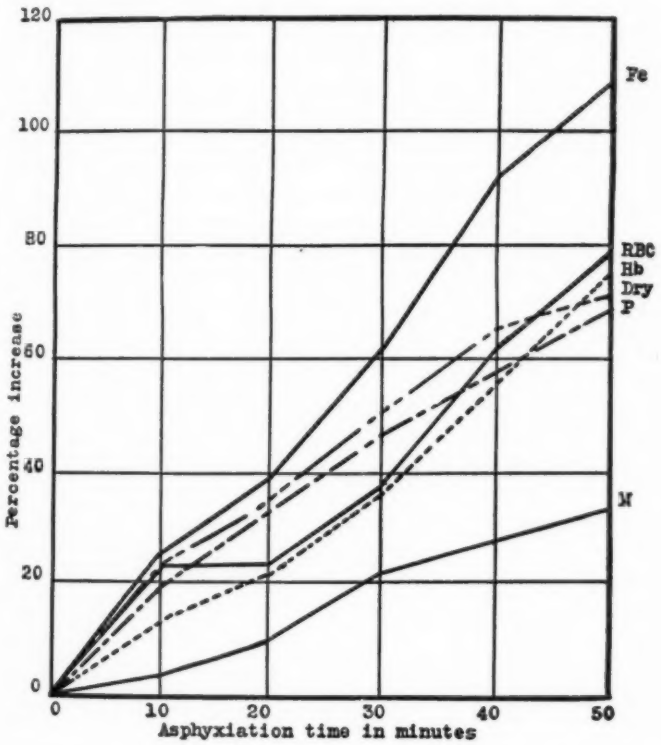

CHART 1. Showing the relative percentage increase in the various blood constituents during asphyxiation.

Oxygen capacity was determined by the Van Slyke method. The new portable manometric blood gas analysis apparatus was employed. No modifications of his method were used excepting that the blood was rotated in a separatory funnel for 10 to 15

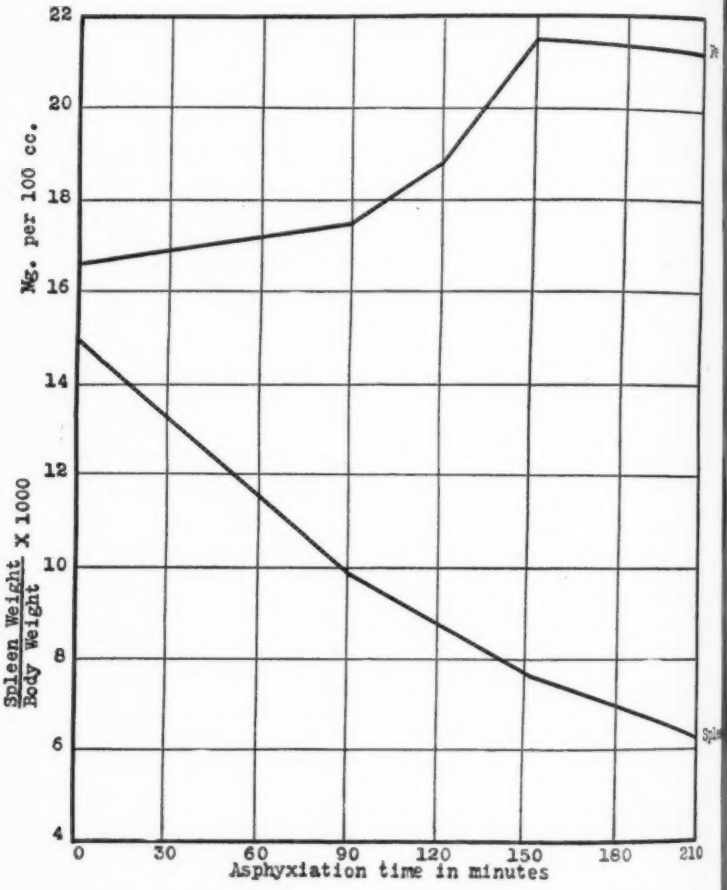

CHART 2. Showing the decrease in the ratio of spleen weight to body weight and the concomitant increase in iron in the blood during asphyxiation.

minutes to insure complete equilibration of the blood gases. 2 cc. of blood were used for samples and checks were nearly always made.

Results.

The results obtained from the analyses of the blood of 100 menhaden at different degrees of asphyxiation are shown in Chart 1. The graph is plotted to show the relative percentage increase in concentration of the various blood constituents. It will be noted that the constituents with the exception of iron and total nitrogen show similar increases in concentration. Iron shows a greater increase in concentration, while total nitrogen shows a smaller increase. Iron does not increase in proportion to the hemoglobin concentration.

Spleens were removed from thirty puffers during different degrees of asphyxiation. The ratio of the spleen weight to body weight was determined. The concentration of iron in the blood of each fish was determined following splenectomy. The results are shown on Chart 2. It will be noted that the iron rose in concentration during asphyxiation and that the spleen decreased in size concomitantly.

Oxygen Capacity.

While investigating the variations in the constituents of the blood during asphyxia, it was observed that the oxygen-combining power of the blood did not increase proportionally with the iron or hemoglobin. There was only a slight increase in the oxygen capacity of the blood while the iron and hemoglobin analyses showed a marked increase in concentration.

The blood becomes acid during asphyxia (4) and variations in the hydrogen ion concentration of the blood are known to produce variations in the oxygen-combining power of the blood (1). It was therefore thought necessary to alter the reaction of the blood of the asphyxiated fish and test the oxygen capacity under such circumstances. This was accomplished by adding about 5 mg. of sodium bicarbonate to each cc. of blood. Previous to the addition of the bicarbonate, the blood was equilibrated with atmospheric air and an initial sample removed on which a Van Slyke determination was made for oxygen capacity. The bicarbonate was then added, the blood again similarly equilibrated, and a second Van Slyke determination was made.

The results of the experiment are shown in Chart 3. They were obtained from ten normal and twenty-four asphyxiated fish.

The fish were asphyxiated in jars, as has been previously described, at a temperature of 20°. Curve C shows the rise in oxygen capacity during 30 minutes of asphyxiation. The blood of normal fish had an average oxygen capacity of 12.04 volumes

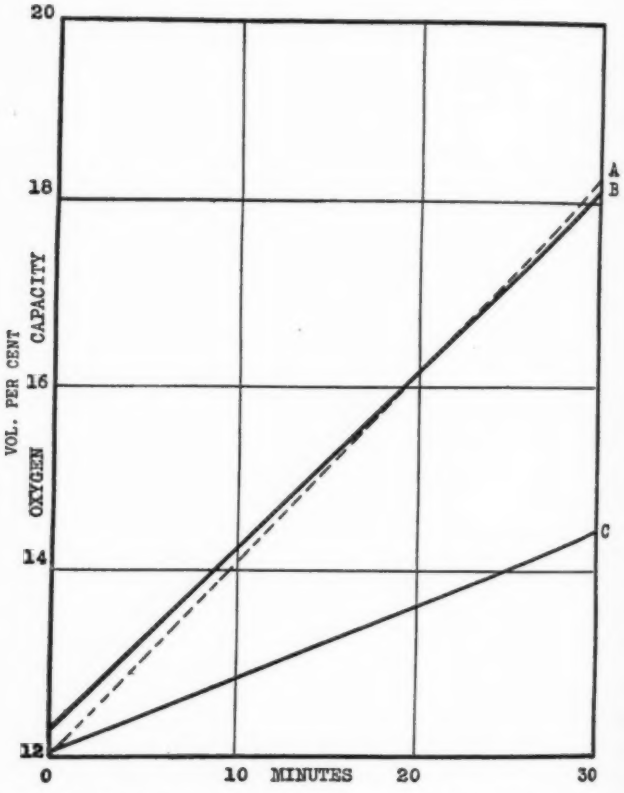

CHART 3. Showing the rise in oxygen capacity of the blood during asphyxiation (Curve C); the rise in oxygen capacity of the blood after the addition of sodium bicarbonate (Curve B); and the theoretical rise in oxygen capacity calculated from the increased concentration of hemoglobin (Curve A). Oxygen capacity is expressed in volumes per cent of blood. The degree of asphyxiation is expressed by time in minutes.

per cent. The blood of the asphyxiated fish at the end of 30 minutes had an oxygen capacity of 14.4 volumes per cent, which indicates a rise from the normal of 24.6 per cent. The same samples of blood showed that the hemoglobin had increased from 8.30 to 12.63 gm. per 100 cc. of blood or an increase of 51.9 per cent in concentration during the 30 minutes of asphyxiation. Curve B represents the average oxygen capacity of the blood to which the bicarbonate had been added. It will be observed that in the blood of the normal fish the bicarbonate produced very little change in the oxygen capacity. The oxygen capacity was increased 0.3 volume per cent. However in the blood of the asphyxiated fish the oxygen capacity was altered from 14.4 to 18.15 volumes per cent at the end of the 30 minute period.

In the blood of a normal fish the ratio of the oxygen capacity to the hemoglobin concentration is very constant, as in other vertebrates. One may plot the theoretical value of one if he knows the value of the other. Curve A, Chart 3, shows such a plotting based upon the concentration of hemoglobin. It represents what the oxygen capacity should be if it were not influenced by some factor produced by asphyxiation. It will be observed that Curves A and B are quite similar, thus indicating that with the addition of the bicarbonate the oxygen-combining power of the blood has been restored to its normal value.

DISCUSSION.

The results obtained in this investigation further corroborate those obtained in a previous paper (5) and indicate that there is an increase in concentration of certain constituents in the blood of fishes during asphyxiation. This increase is mainly due to the release of water from the blood inasmuch as many of the constituents show a relative proportionate increase. Iron, however, does not show an increase proportional to the loss of water from the blood or to the increase in concentration of hemoglobin, but rather an increase at a faster rate. Two hypotheses suggest themselves: (1) that hemoglobin may be broken down in the blood during asphyxiation to free iron, or some intermediary decomposition product of hemoglobin containing iron, or (2) that iron is released from some storehouse of the body in a form simpler than that of hematin. The first hypothesis does not seem

probable for the reason that hemoglobin apparently maintains a fairly constant ratio to the amount of dry matter and phosphorus, and to the number of corpuscles throughout periods of asphyxiation. Evidence in support of the second is shown in Chart 2. It will be observed that the spleen shows a decided decrease in volume which is roughly proportional to the time of asphyxiation. The iron in the blood shows a simultaneous increase in concentration, which is roughly inversely proportional to the change in the spleen volume. It is suggested, therefore, that perhaps the spleen is the storehouse for iron in the body of a fish and that during asphyxiation iron is released into the blood stream.

The oxygen-combining power of the blood did not increase in proportion to the amount of hemoglobin. However, by the addition of sodium bicarbonate into the blood of asphyxiated fish, the oxygen-combining power was brought to a value consistent with the ratio of oxygen saturation to hemoglobin in the normal fish. This indicates that the blood becomes quite acid. Inasmuch as carbon dioxide would be driven off during aeration of the blood the acid is apparently of a fixed nature and probably is the result of metabolic by-products which are not completely oxidized.

The increased concentration of hemoglobin during asphyxia may perhaps be of some adaptive value to the organism since it would compensate to some extent for the loss of its oxygen-combining power. Fishes apparently are able to keep down the concentration of nitrogenous compounds below that of the other constituents during asphyxia. In this way a fish probably protects itself against an accumulation of toxic substances.

SUMMARY.

1. In menhaden and puffer fish asphyxiation produces a definite increased concentration of hemoglobin, phosphorus, iron, total nitrogen, number of red blood corpuscles, and the percentage of dry matter. The increase is roughly proportional to the length of time of asphyxiation. Iron increases at a faster rate and total nitrogen at a slower rate than the other blood constituents.

2. The spleen shows a decrease in size during asphyxiation. It is suggested that in fishes the spleen is a storehouse for iron, which is released into the blood during asphyxiation.

3. The oxygen-combining power of the blood is increased during

asphyxiation but not in proportion to the amount of hemoglobin. This is apparently caused by an increase in acidity.

4. Death of fishes during asphyxiation probably is due to the increased acidity of the blood and tissues and to the retention and concentration of toxic substances.

5. The transfer of water from the blood during asphyxiation may in some conditions be of adaptive value to fishes.

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RICKETS IN DOGS.

METABOLISM OF CALCIUM AND PHOSPHORUS.

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(Received for publication, January 6, 1928.)

For the production of experimental rickets, rats have been the animals most generally used. Mellanby (1, 2) and Jones (3) are the only recent investigators who have experimented extensively on dogs. Mellanby chose them because the course of rickets in puppies more closely resembles that in human beings. He maintains that this disease can be produced by the absence of vitamin alone, regardless of the calcium and phosphorus content of the diet.

It was our purpose to observe the metabolism of calcium and phosphorus in puppies on a rickets-producing diet, from an early stage until rickets developed. Diagnosis was established, as in Mellanby's study, by the appearance of the animal, radiographs, histological examination of the bones, and analysis of them for calcium. In addition, the calcium and inorganic phosphorus of the blood serum were measured in the food, urine, and feces, calcium and phosphorus were determined quantitatively, and the balances of these elements calculated.

Plan of Experiment.—Four mongrel puppies of a litter born September 20, 1925, were taken at the age of 1 week from the mother, which was a pet mongrel bitch of terrier type, weighing about 12 kilos. It was kept in a country place and allowed to run free. Its diet was mixed, principally table scraps. The young were not all of the same type. Three were medium sized long haired pups resembling their mother; the fourth, smaller and more dapper, was short haired.

The pups were raised on the bottle with cow's milk and 5 cc. of cod liver oil daily. 1 week before the experimental period three

XUM

of the puppies were given a diet consisting of 10 gm. of dried skim milk powder, 40 gm. of Quick Quaker Oats, cooked 5 minutes, 5 cc. of linseed oil, and 1 gm. of NaCl. This amount was given for each kilo of weight and equalled 250 calories per kilo. The other dog, the control, received cod liver oil instead of linseed oil. Each dog was given in addition 60 mg. of Harris yeast vitamin concentrate per kilo and daily 5 cc. of orange juice. The food was divided into two portions, one-half given in the morning and one-half in the evening.

Diets.—The diet of milk and oatmeal is relatively rich in calcium and poor in phosphorus. It was selected primarily because we desired to duplicate Mellanby's work (1, 2). Its Ca:P ratio is 0.66. Similar diets were used at the Lister Institute to produce rickets in rats, but the pathological condition which ensued was mild and the degree varied in the different animals. Mellanby does not claim that the diet produced the defect in every case, but it certainly produces severe rickets in dogs.

On November 8, when the puppies were 8 weeks old, they were placed in metabolism cages which permitted separation of urine and feces, and the excreta were saved from this date for the subsequent 10 weeks. Specimens from two of the dogs for the 1st week were lost; for these two dogs, the material studied represents the last 9 weeks. The experiment proceeded without other known errors. The same diet was given throughout, but only 225 calories per kilo were given during the 5th week and 200 calories per kilo for the last 5 weeks. All of the food was eaten. After the experimental period the dogs were given various additions of mineral salts to produce tetany. As the food was no longer eaten quantitatively the excreta were not analyzed. The animals were killed 2 months later, and the bones studied histologically and chemically. The diets had not been greatly altered, and the changes were not designed to cure the ricketic process. Histological and chemical studies of the bones were made only as confirmation of the x-ray findings and blood studies.

Methods.—The feces were formed. They were dried on the steam bath with alcohol, ground, weighed, and sampled. The urine was preserved with thymol-chloroform, made to volume, and an aliquot was taken for analysis. Food, urine, and feces were analyzed for calcium and phosphorus. They were ashed

separately with HNO_3, and the ash was neutralized to methyl red. The calcium was precipitated as oxalate and titrated with permanganate. The phosphate was determined by titration of the yellow precipitate,—a slight modification of Bang's method was used (4). The blood serum was analyzed for calcium by Clark and Collip's modification (5) of the Kramer-Tisdall method (6).

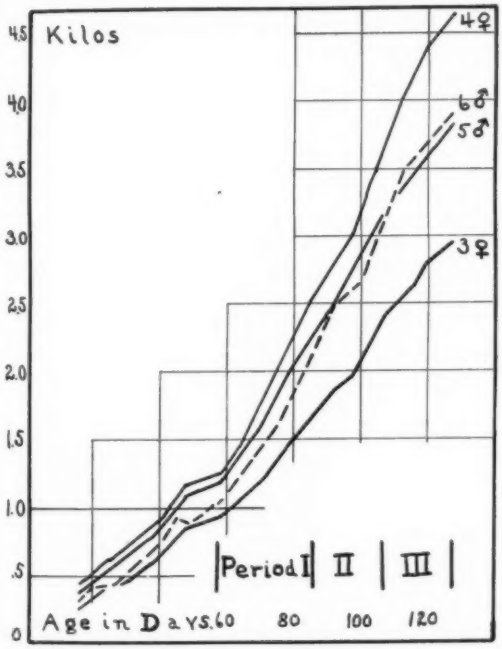

FIG. 1. Growth curves of dogs on a rickets-producing diet. The control dog received cod liver oil instead of linseed oil.

Inorganic phosphorus in the serum was determined quantitatively, by Briggs' method (7). The x-ray pictures were made at the New Haven Hospital every week by courtesy of Mr. E. Furbish. Through the kindness of Miss D. Jackson, sections of the bones were decalcified in Müller's fluid and sectioned and stained with hematoxylin and eosin.

Results.

By all diagnostic standards, marked rickets developed in two of the three dogs which received no cod liver oil. The third dog, Dog 3, the runt of the litter, was the most active, grew the least, and had bones that showed only slight rickets. The control, which received cod liver oil, had practically normal bones. It showed laxity of the tendons, mentioned by Mellanby (1, 2), and the wrists bent under its weight. The two ricketic dogs were less

FIG. 2. Radiograph of Dog 4 on rickets-producing diet. The control dog received cod liver oil instead of linseed oil. Wide metaphyses of advanced rickets are seen. × one-half.

active than the control and the small dog which escaped marked rickets. They all grew well (see Fig. 1) and were in excellent condition at the end of the experiment.

The appearance of the two animals diagnosed as ricketic was similar to that described by Mellanby and figured by him ((1) Fig. 2). The ricketic rosary was marked, and the wrists were enlarged. On section, the fresh long bones showed marked ricketic metaphyses, over $\frac{1}{2}$ inch long and 1 inch wide. These appeared to be composed of cartilage and shelled out in one piece. The

photographs of the fresh bones, the analysis of the bones for calcium, and photomicrographs of the bone sections are not offered as evidence because they were obtained 2 months later and have no quantitative significance for the condition at the time of the experiment. They show that although some slight healing had taken place, severe rickets was still present in the two dogs. The third showed slight rickets, and the control had practically normal bones.

The radiograph taken at the end of the experiment (see Fig. 2) brings out very clearly a condition of severe rickets in Dog 4. There were similar changes in Dog 5, slight changes in Dog 3, and good calcification in the control. The condition shown by x-ray is rickets, as advanced as any shown by Mellanby, and resembles closely his radiographs of the dogs after 9 weeks on a diet which contained a liberal supply of oatmeal ((2), Fig. 2, Experiment 509).

TABLE I.

Blood Serum Analyses of Dogs on Rickets-Producing Diet of Skim Milk, Oatmeal, Linseed Oil, and NaCl.

Days of experiment.	Dog No.	Ca	P	Remarks.
Jan., 1926		*mg. per cent*	*mg. per cent*	
9	4	6.0	4.7	Advanced rickets.
14		7.7	5.6	
9	5	6.7	3.9	Advanced rickets.
16		6.1	5.4	
9	3	11.0	5.1	Mild, healing rickets.
16		9.0	5.5	
9	6	10.1	5.4	Control, normal.*
16		10.2	5.3	

* The control dog received cod liver oil instead of linseed oil.

Blood examinations were made in the third period only, to avoid introducing a factor of anemia. The findings, which are given in Table I, are typical of low calcium rickets for Dogs 4 and 5 and are normal for the other two. The blood findings, the gross appearance of the animals, the bones, the bone analyses, and the radiographs, clearly establish the diagnosis of rickets.

Metabolism of Calcium and Phosphorus.—The calcium and phosphorus balances which accompanied these changes, measure the effect of rickets upon the mineral metabolism. The periods during which metabolism studies were conducted are indicated in Fig. 1. Growth was uniform and satisfactory throughout. Dog 3 grew less rapidly than its litter mates, but was apparently a smaller type. The weights of the dry feces for the experimental periods are given in Table II. The data show that those dogs (Nos. 4 and 5) which became ricketic, produced larger amounts of feces.

Calcium and phosphorus values of the food, urine, and feces, and the balances are given in Table III. They represent three

TABLE II.

Weight of Dried Feces of Dogs on Rickets-Producing Diet.

Calculated per dog per day.

Dog No.*	Period No.		
	I	II	III
	gm.	*gm.*	*gm.*
4	8.7	18.7	20.1
5	7.2	14.3	20.4
3	8.6	11.8	13.6
6	5.9	12.6	15.5

* The control dog received cod liver oil instead of linseed oil.

periods of 3 weeks each (except for Dogs 4 and 5, which had 4 weeks in the first period) calculated in terms of 1 day. The weight of the dogs for each period was taken as the average of the initial and final weight. The dogs weighed slightly differing amounts. The values have been reduced to represent the metabolism per kilo of weight in order to afford a comparison of the separate periods and of the individual dogs.

Paths of Excretion.—Calcium was present in the urine in such small amounts that it could not be determined accurately and is negligible. Phosphorus in the urine averages 35 per cent of the excretion for all four dogs. The control does not differ markedly. This percentage differs from those previously reported for two normal infants (8) on a milk diet, who excreted two-

thirds of the phosphorus in the urine. The urinary phosphorus of rats on a high calcium-low phosphorus diet was, however, only 5 per cent (9). The amounts of calcium and phosphorus excreted in the urine and stool respectively are practically the same in the normal and ricketic subject. The proportions excreted by way of the urine and stool depend primarily upon the composition of the diet.

TABLE III.

Metabolism of Calcium and Phosphorus on Rickets-Producing Diet.

Mg per day; calculated per kilo of dog.

Dog No.*	Period No.	Calcium.				Phosphorus.						Ca:P
		Intake.	Stool.	Balance.	Per cent.	Intake.	Urine.	Stool.	Total.	Balance.	Per cent.	
		mg.	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*	*mg.*	*mg.*		
4	I	118	36	82	69	177	37	82	119	58	33	1.4
	II	100	55	45	45	155	50	100	150	5	3	9.0
	III	102	57	45	44	155	57	79	136	19	12	2.4
5	I	124	41	83	67	188	45	82	127	61	32	1.4
	II	100	41	59	59	152	60	86	146	6	4	9.8
	III	102	79	23	23	155	65	110	175	−20	−13	∞
3	I	123	47	76	62	185	31	100	131	54	29	1.4
	II	100	39	61	61	150	38	89	127	23	15	2.7
	III	102	37	65	64	153	47	72	119	34	22	1.9
6	I	119	24	95	80	179	45	60	105	74	41	1.3
	II	105	24	81	77	174	53	74	127	47	27	1.7
	III	102	24	78	77	155	38	63	101	54	35	1.4

* The control dog received cod liver oil instead of linseed oil. For time of periods see Fig 1.

Balance of Calcium and Phosphorus.—The balance of calcium of all dogs was positive in all periods. The two ricketic dogs retained 53 and 50 per cent of the calcium intake; the dog which failed to develop rickets, on the same diet, retained 62 per cent and the control 77 per cent of the intake. The dogs received cod liver oil before the experimental period and some of the vitamin was probably stored, as is indicated by the difference between the

first period and the two subsequent periods in which the balances are less favorable. If, therefore, the first period is omitted, the values of retention become reduced to 45 and 42 per cent for the two ricketic dogs and remain practically the same for the two other puppies. This shows strikingly that the development of rickets, even on a low calcium diet, is associated with diminished, but not negative balances of calcium.

The balances show a greater deficiency of phosphorus than of calcium. One of the two ricketic dogs showed a negative balance in the third period. The average retentions corresponding to the calcium values given above are 16.2 and 8.5 per cent for the two ricketic dogs, 22.6 for the third puppy, and 34.5 per cent for the control. The averages for the last two periods are 7.8, −4, 19.2, and 31.2 per cent respectively. Thus the balances of calcium and especially of phosphorus are larger for the control than for the ricketic dogs.

The deficiency of phosphorus is further corroborated by the ratio of the retentions of calcium and phosphorus. The normal proportion of calcium to phosphorus retained was shown to be 1.58 for the rat (10). For normal infants, the value calculated from many published data closely approximates this. It may be assumed for our purpose to be similar for the dog also. Higher ratios represent excess calcium retention; lower ratios represent excess phosphorus retention. As shown in Table III, the two ricketic dogs, except for the first periods, showed an increased proportion of calcium retained. The third dog and the control represent calcium and phosphorus retention in normal proportions.

DISCUSSION.

The metabolism data show marked differences between the control which received cod liver oil and the animals on the same diet, which received a fat without vitamin. Two of the experimental animals developed marked rickets. That the third did not develop severe rickets as measured by x-ray and blood findings may be due to the preliminary treatment with cod liver oil, or to the animal's failure to gain as rapidly as its litter mates, or to species difference. The bones, however, showed some evidence of rickets when examined histologically. The study of the metabolism indicates a condition midway between that of the ricketic

animals and that of the control. These data do not measure a qualitative difference but indicate a quantitative measurement of the defect in calcium and phosphorus metabolism. They are preferable, even, to an analysis of the bones, for these may show calcification at the expense of the rest of the body, but the metabolism of calcium and phosphorus measures this defect in the whole organism.

The study of the metabolism shows that development of severe rickets is associated not with a negative balance, but with a diminished positive balance of both calcium and phosphorus, as has been previously shown with the rat (9).

These data show that it is important to measure not only the calcium, but also the phosphorus, and to calculate the relationship between the amounts of these two elements retained. Although in this case the phosphorus in the food exceeded the calcium, the phosphorus balance was smaller or even negative. This indicates an exception to the law that the element of which least is consumed is the limiting factor in nutrition. Here the limiting essential lies in the unknown constituents of the diet. The action of vitamin D thus indicates a new mechanism in the stabilization of calcium and phosphorus metabolism.

Our data also point strongly to the undue and incorrect emphasis laid upon calcium in contradistinction to phosphorus in ossification. The process is universally referred to as calcification. The more important defect in rickets lies in the inadequate retention of phosphorus.

SUMMARY.

1. The study of the metabolism of calcium and phosphorus in puppies on a rickets-producing diet gives a quantitative measure of the degree of abnormality.

2. The calcium balances are positive but less than normal. The phosphorus balances are still more deficient and may be negative.

3. Even on a diet high in phosphorus and low in calcium the most marked deficiency lies in the phosphorus retention.

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THE INFLUENCE OF PHLORHIZIN UPON INORGANIC METABOLISM.

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Considerable interest has been shown in an apparent relationship between the metabolism of carbohydrate and that of some of the inorganic constituents. Work of Wigglesworth, Woodrow, Smith, and Winter (1), Harrop and Benedict (2), Briggs, Koechig, Doisy, and Weber (3), Bolliger and Hartman (4), and others, has shown phosphorus and, in some instances, potassium to be affected, at least as far as blood and urine concentrations are concerned, both by conditions promoting the storage or utilization of carbohydrate (insulin administration, *etc.*) with or without a lowered blood sugar, and by conditions found in depancreatized animals and severely diabetic patients, in which greatly decreased carbohydrate utilization gives rise to the familiar hyperglycemia and glycosuria. A study of the inorganic metabolism of the phlorhizinized animal, whose condition is associated with a lowered blood sugar due to the renal effect of the drug, should throw additional light on the behavior of inorganic metabolites during deranged carbohydrate metabolism, and perhaps on the mechanism of phlorhizin diabetes.

Aside from the observation by Allan, Dickson, and Markowitz (5) of increased urinary phosphorus of the fasting phlorhizinized dog, the literature is of little help in the present problem. Ets (6), who worked with blood during phlorhizin administration, reported only the lipoid and nitrogenous constituents.

EXPERIMENTAL.

Since no significance was to be placed on the actual amount of carbohydrate excreted, and since the very high catabolism of

tissue protein that accompanies the usual phlorhizin administration to fasting animals was an undesirable condition for this work, it was arranged to feed the animals a diet which would spare the tissue protein to a great extent, and thus bring about more comparable conditions throughout a somewhat prolonged experiment. For this purpose meat has the advantage over carbohydrate and fat of being readily eaten by the animals, and, according to Benedict and Osterberg (7), of abolishing the ketosis.

Throughout the experiment, normal female dogs, kept in metabolism cages, were fed a daily ration of 300 gm. of raw lean beef, to which had been added 10 gm. of acid-washed infusorial earth to insure urine collection uncontaminated by feces. When approximately constant body weight had been attained on this diet, the normal period was started, and 24 hour urine quantities were separated by catheterization. This period lasted 7 days, and was followed by a 7 day phlorhizin period, identical with the former, except for the subcutaneous injection at 24 hour intervals of 1 gm. of phlorhizin suspended in 10 cc. of olive oil. An after period of 7 days without the drug followed those described.

The physical condition of the dogs during the phlorhizin period was all that was expected, for while being far from active, there was none of the excessive weakness encountered in animals fasted for an equal length of time. During the after period the animals returned to the normal activity of the beginning of the experiment, instead of being further weakened as would have been the case had no ration been fed.

At the end of each 7 day period, about 30 cc. of blood were taken from the animal by cardiac puncture without anesthetic, all samples being drawn at the same time of day and at least 14 hours after a meal. The sample for the phlorhizin period was taken 14 hours after the administration of the drug. Each blood sample was divided into three portions, one of which was analyzed for sugar and non-protein nitrogen by the Folin-Wu (8) technique, and for chloride by the Whitehorn (9) method. Sodium, potassium, and calcium in a second portion were determined by the Kramer-Tisdall (10) method, magnesium being estimated from the calcium filtrate by the method of Denis (11). The serum from a third portion was analyzed for inorganic phosphorus by the method of Briggs (12), especial care being taken to avoid hemolysis

and long standing before analysis. All analyses were made in duplicate, and were completed as soon after the drawing of the sample as possible.

The 24 hour urine samples, collected under toluene, were each diluted to a litter, and the daily sugar excretion during the phlorhizin and after periods was determined by the Shaffer-Hartmann (13) method and used as an indication of the progress of the ex-

TABLE I.

Effect of Phlorhizin upon Inorganic Constituents of Blood.

Dog No.	Period.	Weight.	Mg. per 100 cc. blood.							Inorganic P.
			Sugar.	Non-protein N.	Cl	Na	K	Ca	Mg	
		kg.								*mg. per 100 cc. serum*
1	Normal.	8.8	107.0	31.6	323	225	25.0	5.4	3.8	3.70
	Phlorhizin.	8.0	57.1	28.8	317	182	18.0	5.4	4.2	5.31
	After period.	7.5	92.2	30.5	330	184	25.8	5.2	3.4	3.84
2	Normal.	8.5	95.3	30.8	320	176	28.3	5.0	4.0	2.63
	Phlorhizin.	8.1	54.4	30.7	318	177	18.6	5.2	3.8	5.88
	After period.	7.5	105.0	31.5	330	171	25.1	5.1	4.2	2.50
3	Normal.	6.7	109.2	31.3	361	219	23.5	5.6	3.8	2.75
	Phlorhizin.	5.8	64.5	28.7	350	208	20.5	5.3	3.2	4.02
	After period.	4.8	92.0	30.7	357	206	23.6	5.4	3.6	2.81
4	Normal.	10.1	99.8	28.8	288	192	22.1	5.2	3.8	2.51
	Phlorhizin.	8.9	60.2	25.7	280	196	19.0	5.4	4.2	3.12
	After period.	7.9	100.3	28.5	283	193	21.9	5.5	4.2	2.73
Mean.	Normal.		102.8	30.9	323	203	24.7	5.3	3.8	2.89
	Phlorhizin.		59.1	28.5	316	191	19.0	5.3	3.8	4.58
	After period.		97.4	30.3	325	189	24.1	5.3	3.8	2.97

periment. Urines for each of the three periods were then pooled and analyzed for nitrogen and chloride by the familiar Kjeldahl-Gunning and Volhard methods, phosphorus by the Fiske and Subbarow (14) method, sodium, potassium, and calcium by the Kramer-Tisdall (15) method, and magnesium by that of Hammett and Adams (16). The values reported are the average 24 hour excretions for each period, with the exception of the sugar figures, which are the average of the daily determinations for each period.

TABLE II.

Effect of Phlorhizin upon Inorganic Constituents of Urine.

Dog No.	Period.	Weight.	Average 24 hr. excretion.									Ratio.	
			Volume.	Sugar.	N	Cl	P	Na	K	Ca	Mg	P:N	K:N
		kg.	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*		
1	Normal.	8.8	193	0	9.71	0.145	0.48	0.187	0.905	0.018	0.015	0.049	0.093
	Phlorhizin.	8.0	532	32.1	12.20	0.314	0.56	0.240	1.162	0.030	0.026	0.046	0.095
	After period.	7.5	320	7.6	9.60	0.146	0.44	0.232	0.730	0.012	0.019	0.048	0.076
2	Normal.	8.5	186	0	10.30	0.150	0.55	0.149	0.930	0.018	0.027	0.053	0.090
	Phlorhizin.	8.1	421	37.9	11.60	0.109	0.55	0.217	1.263	0.019	0.031	0.047	0.108
	After period.	7.5	307	10.9	10.40	0.190	0.47	0.198	0.881	0.013	0.018	0.054	0.085
3	Normal.	6.7	145	0	8.14	0.145	0.44	0.230	0.895	0.030	0.022	0.054	0.110
	Phlorhizin.	5.8	354	34.6	11.00	0.190	0.64	0.254	1.020	0.030	0.033	0.058	0.093
	After period.	4.8	280	19.1	10.10	0.145	0.53	0.237	1.032	0.029	0.029	0.053	0.103
4	Normal.	10.1	215	0	8.65	0.150	0.47	0.263	0.985	0.023	0.024	0.054	0.114
	Phlorhizin.	8.9	770	45.6	12.50	0.265	0.64	0.297	1.340	0.027	0.028	0.051	0.107
	After period.	7.9	370	15.4	10.00	0.169	0.53	0.281	1.050	0.020	0.020	0.052	0.105
Mean.	Normal.		185		9.20	0.147	0.48	0.218	0.929	0.022	0.022	0.053	0.102
	Phlorhizin.		519		11.80	0.219	0.60	0.252	1.190	0.028	0.030	0.051	0.101
	After period.		369		10.00	0.162	0.48	0.237	0.920	0.018	0.021	0.050	0.092

It will be noted from Table I that, under the existing conditions, after phlorhizin had been administered for a period of 7 days, in every case there were, besides the familiar hypoglycemia, a distinct lowering of the potassium concentration in whole blood and an increase of the inorganic phosphorus of the serum. In the whole blood, chlorine was lowered to a slight extent, while sodium, calcium, and magnesium remained practically unchanged. In spite of the continued loss in weight by the animal throughout the after period, the potassium and phosphorus values returned to those of the prephlorhizin level. Non-protein nitrogen remained approximately constant throughout the three periods.

The urine (Table II) shows an increased excretion of all the inorganic constituents under the influence of the drug, with a return towards normal in the after period. A negative Benedict test showed no sugar being excreted at the end of the experiment when the final blood sample was taken.

When the blood and the urine figures for corresponding periods on the four dogs are averaged (Tables I and II), it will be seen that while sodium, calcium, magnesium, and chlorine in the whole blood remain within 6 per cent of their normal concentrations, the drop in potassium is one of 23 per cent and the rise in inorganic phosphorus of serum one of 59 per cent during the phlorhizin period. Though the urine shows an average increase of 22 per cent in potassium excretion during the phlorhizin period, this is no greater than the increase of other elements which showed no fall in blood concentration. Such increased excretion is due either to diuresis, or, more probably, to the somewhat increased protein catabolism, for the potassium : nitrogen ratio remains nearly constant. Increased blood inorganic phosphorus is accompanied by no increase in the urine above that due to protein catabolism (phosphorus : nitrogen remains constant), nor is there an increased excretion during the after period to show a simple retention in the blood during phlorhizin, due to delayed or difficult excretion. In fact inorganic phosphorus has been shown by Denis (17), and by Addis, Meyers, and Bayer (18) to be normally very rapidly excreted.

DISCUSSION.

It will be noted that, as in other work dealing with deranged or abnormal carbohydrate metabolism, potassium and phosphorus

are the only inorganic metabolites to show appreciable change in the blood. That these should be the chosen among the available anions and cations is suggestive of a definite mechanism in which they have a special function to perform. From the blood and urine findings reported in the present paper it would appear as if potassium, lowered in the blood and not correspondingly raised in excretion, increased in concentration in the tissues during the period of carbohydrate mobilization. Whether inorganic phosphorus in the tissues during this period was larger or smaller than normal would depend upon whether the extra inorganic phosphorus of the blood at that time came from the other blood phosphorus compounds, or from the tissues themselves.

Inorganic blood phosphorus change during phlorhizin poisoning is found to be the same in direction and amount as that observed by Bolliger and Hartman (4) in depancreatized dogs and by Harrop and Benedict (2) in severely diabetic patients, and directly opposite to that reported by Briggs, Koechig, Doisy, and Weber (3) following insulin administration to dogs. It would seem that consideration of the phosphorus behavior would add to the accumulating evidence that phlorhizin poisoning gives rise to a diabetes whose mechanism is not entirely restricted to renal damage but resembles that in which pancreatic hormone is concerned. It is interesting to note that carbohydrate mobilization and excretion, whether brought about by phlorhizin or by insulin deficiency, gives rise to the same change in serum phosphorus though in one instance there is a low blood sugar level and in the other a hyperglycemia.

SUMMARY.

Phlorhizin administered for 7 days to dogs on a constant weighed diet of lean beef produced in the blood a depression of potassium and an elevation of serum inorganic phosphorus. Sodium, calcium, magnesium, and chlorine in whole blood remained unchanged, as did the non-protein nitrogen.

In spite of the continued loss in weight by the animals, the potassium and phosphorus values returned to normal in the after period.

There was no compensating change of potassium or phosphorus in the urine during either the phlorhizin or after periods that was not accounted for by slightly increased protein catabolism.

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THE LACTIC ACID CONTENT OF RESTING MAMMALIAN MUSCLE.

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(Received for publication, December 27, 1927.)

The amount of lactic acid which can be present normally in living mammalian muscle varies between such wide limits that a characteristic value can be obtained only within uniformly well defined experimental conditions. The amount usually found is larger than that actually present in the tissue at the time of killing the animal, on account of the rapid formation during death struggles.

Some of the lowest values previously observed for the lactic acid content of mammalian muscle include one of 11 mg. per 100 gm. of tissue[1] made by Kuhn and Baur (8) on a rabbit which had been allowed to die following fasting, and several of 10 to 14 mg. by Wacker (12). The former obtained 23 mg. as a minimum value after death from insulin overdosage, the latter 38 mg. Similar amounts, 23 and 36 mg., were observed by Hoet and Marks (7) during the immediate rigor following the death of rabbits which had been subjected to excessive thyroid feeding. They obtained 54 mg. in the muscles of an animal which had died from insulin overdosage.

Many of the values found in the literature are considerably higher. The resting values for muscles of rats, which had been kept on a high fat diet and subjected to exercise, are given by Meyerhof (9) as varying from 70 to 140 mg. Embden, Schmitz, and Meincke (2) have found 57 to 79 in phlorhizinized dogs and amounts varying from 46 to 295 in rabbits. Cori (1) observed 83 to 120 in fasted mice, some of which were controls, others insulin-treated, and a third group epinephrine-treated.

The very low values have been associated with depletion of the carbohydrate stores, and represented almost all of the lactic acid which could be formed by the muscle. Incubation (2 to 4 hours) of the muscle whose carbohydrate was depleted was followed by no gain or a very small gain in the lactic acid content found at death (7, 8, 12). This indicates either that the

[1] All values have been expressed as mg. per cent.

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muscle which contains glycogen contains also a fairly large amount of lactic acid while in the living animal, or that much of the lactic acid usually found is produced by the convulsions and anoxemia during death. That the amount of lactic acid in fresh mammalian muscle is largely determined by the method of handling was shown by Fletcher (3). He obtained 80 mg. (as Zn lactate) in the resting muscles from the hind limbs of rabbits by dropping the muscles singly into liquid air and grinding the brittle masses in ice-cold alcohol. As a caution, he states, "It is most important in arranging the technical details of any cooling method to secure that when the muscle has once been frozen there shall be no return to thawing temperature. . . ." His value is higher than those obtained in the carbohydrate-depleted muscle, and since the circulation had stopped, it seems likely that in spite of the excellent technique, a considerable amount of lactic acid formed during the death of the animal.

The ideal procedure for the determination of the normal lactic acid content of resting mammalian muscle would consist of instant freezing *in situ* without any stimulation of the muscle in the living, unanesthetized animal, removal from the body, and stopping enzymatic action either before or just as the muscle thawed.

The values observed by Gasser and Dale (5) for resting gastrocnemii of cats (35 to 66 mg.) were obtained by excising the muscles from living animals, without freezing, and stopping the action of enzymes by grinding in ice-cold hydrochloric acid. We have observed still lower values by the method described below.

Procedure.

Normal, well fed guinea pigs and white rats were used, and the gastrocnemius muscle chosen on account of its accessibility and general use for experimental purposes. The anesthetic was usually amytal[2] (Lilly), but chloral hydrate was used in several experiments. The dosage of amytal, given intraperitoneally, was found to be larger for rodents than for dogs, and from 100 to 150 mg. per kilo of body weight were necessary to give surgical anesthesia. The susceptibility varied, but 60 mg. for a 500 gm. guinea pig was usually successful, although a few died with this dosage. Anesthesia was never quite perfect, and the animals exhibited a few reflex movements during the dissection.

[2] Amytal should be in perfect solution when injected, and it is suggested that a 50 per cent excess of normal alkali be added, and when solution is complete, the excess titrated back to pH 8.5 with 0.1 N HCl.

The gastrocnemii of both sides were dissected free from surrounding structures, but the blood and nerve supply (except when stated otherwise) was left intact. After one side was completed, a ligature was put on the tendon for future convenience in handling, and the skin closed with metal clips. The opposite side was treated likewise and after the dissections were completed the animal was allowed to rest for 5 to 10 minutes. In several experiments the tendon was cut, but this seemed not to affect the amount of lactic acid found. After resting, the skin incision was opened gently and the muscle exposed. The muscle was then frozen as quickly as possible *in situ*, and after freezing solid, removed from the animal and kept in CO_2 snow until sectioned.

Freezing was accomplished (except in two experiments with ethyl chloride) either by packing CO_2 snow around the muscle or with a mixture of CO_2 snow and ethyl chloride.[3] The latter was mixed to form a soft slush, and since it made better contact with the tissue, froze much more rapidly than the snow alone. It usually caused more twitching, however. The CO_2 snow was prepared in the usual manner by allowing the gas to escape from a tank into a bag of several thicknesses of muslin, and after about 25 gm. had formed, the snow was transferred to a small tray made of cork. The ethyl chloride was dropped on until the mass became soft. Freezing of the gastrocnemius of the guinea pig required about 10 seconds with the mixture, and from 3 to 4 minutes with the snow alone.

The rapid destruction of enzymes was accomplished by the following method. The frozen muscle was held on a cork block and cut by hand into slices about 0.1 mm. thick, with an ordinary pocket-knife sharpened to a razor edge, while an assistant dropped the sections singly into a tared vessel (100 cc. extraction flask preferred) which contained 40 cc. of 5 per cent aqueous trichloroacetic acid. The flasks were kept in an ice bath. After reweighing, the volume was made up to 50 cc. for each sample.

Lactic acid was determined by the procedure described by Friedemann, Cotonio, and Shaffer (4). It was found necessary to modify the technical details in order to increase the accuracy for quantities of lactic acid between 0.05 and 0.25 mg. The essential

[3] Ethyl chloride manufactured by Stratford-Cookson Co., Philadelphia.

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changes were the use of a straight glass tube, 300 mm. long and of 6 mm. internal diameter, for the absorption tower (Fig. 1), and the limitation of the amount of fluid used in the titration. No beads were used in the tower, and the tube was cleaned to drain perfectly in order to require a minimum amount of wash water. 2 cc. of

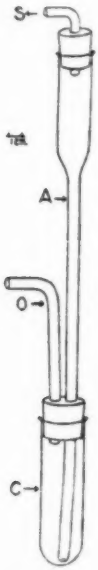

FIG. 1. Type of apparatus used for the absorption of small quantities of acetaldehyde. *S*, suction; *A*, absorption tower; *O*, outlet tube from distilling flask; *C*, 50 cc. centrifuge tube.

1 per cent bisulfite were used for absorption of acetaldehyde. By these changes, the final volume for titration was kept under 5 cc. Larger volumes decreased the sharpness of the end-points.[4]

[4] The free bisulfite was titrated with 0.1 N iodine slightly beyond the first end-point and titrated back with 0.02 N thiosulfate. The final adjustment was made with 0.002 N iodine. From 0.05 to 0.2 gm. of $NaHCO_3$ as the dry powder was used to liberate the bound sulfite. The final titration was made from a 5 cc. micro burette with 0.002 N iodine.

In the determination of very small quantities of lactic acid, the refluxing system should not be allowed to run so cold that traces of acetaldehyde remain dissolved in the water condensed on its surfaces.

A high grade starch indicator is essential.

TABLE I.

Lactic Acid in the Gastrocnemii of Guinea Pigs (Animals 3 to 15) and White Rats (Animals 21 to 25).

Animal No.	Sample No.	Lactic acid.	Protein precipitant.*	Condition of animal and time of freezing.	Method of freezing.
		mg. per 100 gm.			
3	5	69	T.	Dead. Muscles frozen 12 min. post mortem.	Entire leg enclosed in a muslin bag and a jet of CO_2 directed into the bag.
	6	71	S.		
4	7	32	T.	Alive.	
	8	33	S.		
5	9	34	T.	Alive.	
	10	32	S.		
6	11	261	T.	1 hr. post mortem.	
	12	273	S.		
7	13	94	T.	5 min. post mortem.	
8	14	21	T.	Alive.	CO_2 snow packed around muscles.
	15	21	S.		
9	16	13	T.	Alive. Sample 17 removed 1 hr. after Sample 16.	
	17	11	"		
10	18	11	T.	Alive. Sample 19 frozen immediately after Sample 18. Blood lactic acid 8 mg.	
	19	19	"		
11†	20	16	T.	Alive. Both muscles twitched during freezing.	CO_2 snow plus C_2H_3Cl as a slush packed around muscles.
	21	20	"		
12	22	14	T.	Alive. Sample 22 ligated at origin, cut off, and plunged into freezing mixture. Sample 23 frozen *in situ*.	
	23	12	"		
13	24	54	T.	Dead. 2 min. elapsed from stunning to completion of freezing.	
	25	Lost.	"		
14	26	69	T.	Dead. 2 min.	
	27	104	"	" 6 "	
15	28	42	T.	Dead. 1.5 "	
	29	60	"	" 6 "	
21	30	33	T.	Alive. 4.5 min. to complete freezing.	C_2H_3Cl spray.
	31				
22	32	53	T.	Alive. C_2H_3Cl dropped on the muscle for about 6 min.	C_2H_3Cl in a current of air.
	33	47	"		

TABLE I—*Concluded.*

Animal No.	Sample No.	Lactic acid.	Protein precipitant.*	Condition of animal and time of freezing.	Method of freezing.
		mg. per 100 gm.			
23	34	15	T.	Alive.	Packed with CO_2 snow.
	35	16	"		
24	36	19	T.	Alive.	CO_2-C_2H_3Cl slush. Considerable twitching in all four muscles.
	37	24	"		
25†	38	24	T.	Alive.	
	39	27	"		

* T. = trichloroacetic acid. S. = Schenck's reagent.
† Anesthetized with chloral hydrate.

A series of sixteen trials with a standard zinc lactate solution gave apparent recoveries of 95 to 116 per cent. Values above 105 per cent were seen only with quantities of lactic acid less than 0.1 mg., and the tendency of the method was toward a higher apparent recovery as the quantity taken was further decreased.

After the tissue had been fixed and allowed to come to equilibrium with the surrounding fluid, the fluid was filtered off and neutralized to about pH 5.0 with 50 per cent NaOH solution. An aliquot was taken and 2.5 cc. of 20 per cent $CuSO_4$ added. Finely powdered $Ca(OH)_2$ was added according to the Salkowski-Van Slyke procedure as recommended by Cori (1) for the removal of interfering substances from tissue filtrates. The mixture was centrifugalized and an aliquot taken for lactic acid determination. All aliquots were made as large as possible since the determination of less than 0.05 mg. of lactic acid was considered unreliable.

In some preliminary experiments, Schenck's reagent was used as a protein precipitant and duplicate determinations with it and trichloroacetic acid were compared. The results checked provided that both filtrates were treated with $CuSO_4$ and $Ca(OH)_2$. A lactic acid standard to which was added a quantity of trichloroacetic acid equal to that used in the experiments gave the usual value, hence it seemed that trichloroacetic acid did not interfere. Samples 5 to 15 inclusive (Table I) show comparative data obtained from samples made nearly identical by putting alternate slices into

two flasks, one containing Schenck's reagent, and the other trichloroacetic acid. Samples 16 to 39 represent separate muscles.

Animals 13 to 15 were killed by a blow on the head and the muscles removed from the body before freezing.

DISCUSSION.

The amounts of lactic acid found were definitely lower than those previously recorded for the lactic acid content of resting mammalian muscle. We believe that the method of obtaining the muscle was responsible to a considerable extent for the low values,[5] but the possibility that amytal has a depressing effect upon the amount of lactic acid present in muscle during life must be considered. However, the muscle is capable of forming lactic acid rapidly after death as is shown in Animals 3 and 7. The muscles of animals anesthetized with amytal are very flaccid, hence with the circulation of the blood intact, the aerobic type of metabolism predominates, and the substance generally considered to be typical of anaerobic metabolism is present in a very small amount.

The values obtained with amytal anesthesia are similar in magnitude to those obtained for resting muscles of frogs, and also for the lactic acid content of the blood of resting human beings. Hill, Long, and Lupton (6) and Ronzoni and Wallen-Lawrence (11) have demonstrated the free diffusibility of lactic acid from muscles into the blood stream by analyses of blood taken immediately after exercise. Therefore, it seems likely that in the resting state, the lactic acid content of the blood is in equilibrium with, or perhaps somewhat lower than that of the musculature.

At first thought it would seem that freezing a muscle very rapidly with ethyl chloride-CO_2 slush should give lower lactic acid values than slower freezing with CO_2 snow alone. The former cause considerably more twitching so probably raised the lactic acid content before freezing was complete. Slow freezing by ethyl chloride alone was unsatisfactory as shown by Rats 21 and 22.

[5] Experiments now in progress with cats seem to indicate that the lactic acid level both in muscle and blood is markedly higher during ether anesthesia than it is during amytal anesthesia. These observations agree with the findings of Ronzoni, Koechig, and Eaton (10) that ether anesthesia causes an acidosis of the lactic acid type.

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Freezing the leg of the animal in a cloth bag by means of a CO_2 jet was unsatisfactory also. In two preliminary experiments, not included in Table I, values from 50 to 55 mg. were obtained by this method of freezing. The mechanical stimulation of the muscle by the small particles of snow shot out at high velocity by the jet could account for the high values. Rat muscle appeared to be more irritable to mechanical stimulation and stimulation by rapid change of temperature than guinea pig muscle.

SUMMARY.

1. The lactic acid content of resting guinea pig and rat muscle was found to be about 15 mg. per cent.

2. The undesirability of killing animals before removal of muscles for lactic acid determination was shown by the high values obtained by that method.

3. Freezing mammalian muscles *in situ* with their circulation intact and stopping enzyme action during thawing is the logical procedure in preparing them for the determination of resting lactic acid content.

4. Reasons for believing that amytal anesthesia does not depress the formation of lactic acid in muscle, other than by inducing complete rest, are given.

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DIETARY REQUIREMENTS FOR FERTILITY AND LACTATION.*

XIII. STORAGE OF FAT-SOLUBLE VITAMINS FOR LACTATION, WITH SOME OBSERVATIONS ON THE COD LIVER OIL REQUIREMENTS OF NURSING YOUNG.†

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In planning the experiments of this investigation the author had it as his objective to obtain convincing evidence for the existence of a specific vitamin (other than vitamins A and D) for lactation. Although the extensive data submitted in previous publications (1) were very suggestive, he has not been convinced that he has clearly demonstrated that vitamin E is a composite of two specific fat-soluble vitamins, one essential for fertility and the other for lactation. It was anticipated that further progress could be made in obtaining information towards a solution of this intricate problem by depleting lactating females from our stock colony receiving Stock Diet 1 (2) of all reserves of fat-soluble vitamins, and then employing the curative method at the point of failure of the young by vitamin administrations to either mothers or litters. Having already determined that the vitamin B requirements for normal lactation are considerably greater than for normal growth (3),

* The term "reproduction," defined by the author (4) to include lactation as well as fertility, is still accepted by the majority of investigators of nutrition to apply only to fertility. Since my researches, while dealing with the problem of fertility, are mainly concerned with the problem of lactation, the title of this series of investigations has been changed from "Dietary Requirements for Reproduction" to "Dietary Requirements for Fertility and Lactation."

† Research paper No. 57, Journal Series, University of Arkansas.

the experimental rations on which the mothers with their litters were transferred were supplied with an abundance of yeast. The results of the experiments are given in the accompanying tables.

In previous work on lactation we were handicapped by lack of specific knowledge of daily food consumption during the critical stages of lactation when infant mortality is encountered. In this communication and the papers following daily food consumption records are submitted. We were very much surprised to find that the females transferred from Stock Colony 1, receiving a cereal ration containing 1 per cent of cod liver oil (2), to a purified synthetic fat-soluble vitamin-deficient diet without exception reared all their litters at a normal rate. The next procedure was to develop a stock colony on a ration allowing less storage of fat-soluble vitamins. For that purpose the simplified diet of Sherman and Muhlfeld (5), consisting of whole wheat 66.0, whole milk powder 32.7, and NaCl 1.3, was adopted. The latter diet we have designated as Stock Diet 2, and the colony developed on that diet, Stock Colony 2. It was calculated that this diet, containing 32.7 per cent dried whole milk, carries approximately 9 per cent milk fat, and since butter fat is now generally accepted as being considerably inferior in the potency of both vitamins A and D to cod liver oil, it was anticipated that we should have no difficulty in depleting nursing mothers of fat-soluble vitamins when transferred from the whole wheat-milk powder diet (on the date of birth of the young) to purified fat-soluble vitamin-deficient diets. Contrary to all expectations, this was not the case. We have, therefore, continued our experiments by taking females of the *second* generation from Stock Colony 2, and for the first time we began to encounter ophthalmia among numbers of litters. Even among individual females of the second generation we have found considerable storage, but on continuing the young on their mothers' experimental diets, mild to severe ophthalmia developed within 4 to 14 days. It will be noted from Tables I to IV that not only did the mothers carry enough reserves of fat-soluble vitamins from their previous dietary for the requirements of the nursing young, but they themselves lost very little weight. In striking contrast to this it will be shown in Paper XIV that when females from Stock Ration 1 are transferred to vitamin B-deficient diets, they sacrifice 20 to 30 per cent of their body weights in order

to prolong lactation. At a critical stage during lactation vitamin B must be supplied to avoid mortality to mother and young. Although, as is brought out in Paper XIV, yeasts from different sources prepared according to different methods, vary considerably in their potency as a source of vitamin B for lactation, when large proportions are furnished in the ration we found very little differences in the lactation efficiency index upon employing the various types of yeasts used in this investigation.

After rebreeding the females that have, because of the storage of fat-soluble vitamins, reared their first litters on the fat-soluble vitamin-deficient diets, we have encountered in most cases sterility, and in cases where second litters were born the majority of the young died some time during the nursing period. In outlining these experiments they were so planned as to show, if possible, whether vitamins A and D are also essential for fertility and lactation. Although, as it will be brought out later, we have not as yet succeeded in settling the matter of the specificity of a fat-soluble galactagogic vitamin, other than vitamins A and D, we now have clear evidence that diets abundant in vitamin E, but deficient in vitamin A, also produce female sterility characterized by a resorption of the fetus during gestation, as is evidenced by the resorption curve and photograph of the uterine horns (6). The results of these investigations will be published elsewhere. This communication is limited to the lactation performance of first litters when their mothers are transferred from a known previous dietary régime to fat-soluble vitamin-deficient diets.

DISCUSSION.

For some time we had encountered so much difficulty in the depletion of fat-soluble vitamins in lactating mothers transferred from Stock Colonies 1 and 2 to fat-soluble vitamin-deficient rations that, when we finally succeeded in doing so, the author was at first inclined to ascribe the phenomenon to a possible error in compounding the rations. The diets were, therefore, made up anew and fresh yeast used, but there was no response in the growth of the nursing young. It was then suspected that we still were not supplying enough of vitamin B, but Table I shows clearly that Females 4679 and 4680 showed no response to supplementary

TABLE I.

Lactation Record of Females 4679 and 4680 (Second Generation) Transferred from Stock Diet 2 on Date of Birth of First Litters to Fat-Soluble Vitamin-Deficient Ration 1093.

Composition of Ration 1093: casein (purified according to the method of Osborne and Mendel, by repeated extraction with hot 95 per cent alcohol) 20, Northwestern yeast 10, McCollum's Salt Mixture 185 (7) 4, dextrin 66.

Date.	Female 4679.*	Litter.	Female 4680.*	Litter.
May, 1927	*gm.*	*gm.*	*gm.*	*gm.*
25	218			
26	184	30	260	
27	182	34	190	34
28	186	38	190	40
29	189	42	200	43
30	196	50	195	53
31	200	58	198	58
June				
1	197	64	199	67
2	200	71	199	75
4	198	86	198	93
6	190	101	190	111
8	187	110	197	127
10	175	113	188	138
14	167	117	182	151
16	162†	122	174	155‡
17	159§	121	169§	149
18	158	123‖	171	148‖
19	160	126‖	171	170‖
20	176	139‖	178	189‖
21	180¶	149‖	182¶	200‖
22	180	159‖	186	214‖
23	190	172**	190	240**††
24	180	177	191	258
25	190	190	192	274
26	189	200	191	285
27	190	207	190	304
28	193	217	187	311
29	192	224	190	333
30	194	226	188	340
July				
1	192	234	185	345
2	192	243††	182	346
5	190	270	170	380

TABLE I—*Concluded.*

Date.	Female 4679.*	Litter.	Female 4680.*	Litter.
July, 1927	*gm.*	*gm.*	*gm.*	*gm.*
7	193	279	165	398
8	190	287	167	412
9	189	296	165	445

* Females 4679 and 4680 had litters of seven and ten young respectively and were allowed six each to rear.

† 500 mg. of Yeast Concentrate P-19619 to Female 4679.

‡ 500 mg. of Yeast Concentrate P-19619 to litter of Female 4680.

§ 500 mg. of Yeast Concentrate P-19618 to Females 4679 and 4680.

‖ 0.1 cc. of cod liver oil to each young of both litters (separately from the ration); yeast administration discontinued.

¶ Mothers separated from litters for 1 hour just preceding cod liver oil therapy.

** Cod liver oil administration discontinued for the rest of the experiment. Litter of Female 4680 weaned.

†† Litter weaned.

separate administrations of concentrated preparations from yeast.[1] There will be noted, however, the remarkable response of the nursing young to cod liver oil administrations.[2] Five daily administrations of 0.1 cc. per young was by far more than sufficient for weaning of the litters. Since it is quite apparent that the litters of Females 4679 and 4680 have, by receiving a total of 0.5 cc. of cod liver oil (per nursing young), stored appreciable amounts of fat-soluble vitamins, we next proceeded to investigate the minimum amounts of cod liver oil necessary to be supplied to nursing young during lactation for weaning, employing the curative method when the fat-soluble vitamin reserves in the lactating mother showed depletion.

Since we found in our laboratory that by drawing out a medicine dropper to a fine capillary it is possible to give baby rats at birth a small drop of water, we next proceeded to attempt to administer at birth a small drop of cod liver oil, which we discovered they take very readily. Such findings enabled us to proceed with cod

[1] The methods of preparation of the yeast concentrates are described in Paper XIV, following.

[2] We have used the same brand of cod liver oil, deficient in vitamin E, employed by us since 1921, and secured from the Seapure Products Company, Portland, Maine.

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TABLE II.

Lactation Record of Females 4733 and 4734 (Second Generation) Transferred from Stock Diet 2 on Date of Birth of First Litters to Fat-Soluble Vitamin-Deficient Ration 1025.

Composition of Ration 1025: casein (purified by repeated extraction with hot 95 per cent alcohol) 20, Harris yeast 10, McCollum's Salt Mixture 185 4, agar-agar 2, dextrin 64.

Date.	Female 4733.*	Litter.	Food consumed during previous 24 hrs.	Female 4734.*	Litter.	Food consumed during previous 24 hrs.
June, 1927	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
29	255			210		
30	271		10	234		11
July						
1	220	27(6)†	15	186	28(6)†	11
2	200	26	17	190	28	15
3	201	28	23	193	31	23
4	208	30‡	23	197§	30(5)†	23
5	214	34	13	197	34	13
6	217	37	31	200	37	30
7	222	43	23	202	43	17
8	225	52	36	199	52	27
9	229	61	30	197	60	24
10	228	69	20	203	67	17
11	232	80	39	201	76	29
12	230	93	26	198	83	20
13	233	101	28	200	89	24
14	233	113	26	206	96	22
15	231	124	35	204	102	28
16	232	130	28	205	110	29
17	229	141	23	205	117	18
18	228	150‖	36	208	120‖	31
19	224	155	25	202	127	23
20	228	160	28	205	132	25
21	224	160	29	202	135	27
22	220	168	21	203	141	22
23	218	168	14	204	145	23
24	212	184¶	21	202	157¶	32
25	218	204	40	204	170	43
26	214	214	32	202	180	28
27	218	228	43	206	188	30

TABLE II—*Concluded.*

Date.	Female 4733.*	Litter.	Food consumed during previous 24 hrs.	Female 4734.*	Litter.	Food consumed during previous 24 hrs.
July, 1927	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
28	216	234	40	203	185	28
29	219	242**	38	206	192	
30				207	204**	

* Females 4733 and 4734 had litters of thirteen and six young respectively and were allowed six each to rear.

† Figures in parentheses represent number of young left on date indicated.

‡ 1 small drop of cod liver oil (12 mg.) administered to each young, separately from the ration, with a medicine dropper. This is the only day throughout the lactation period the litter received cod liver oil therapy.

§ 6 small drops of cod liver oil to Female 4734. This is the only administration of cod liver oil this mother received during the entire nursing period.

‖ Young opened their eyes.

¶ Young eating.

** Young weaned.

liver oil therapy early in lactation at critical stages, to avoid early infant mortality produced by possible deficiencies of vitamins A and D in the diet of the nursing mother. The litter of Female 4733 (Table II) offers a clear illustration. After having gained only 3 gm. during a period of 72 hours, each young, weighing 5 gm., was given with a medicine dropper a small drop of cod liver oil equivalent to 12 mg. 24 hours after the cod liver oil therapy the litter gained 1 gm. more than during the preceding 72 hours. Since this is the only cod liver oil administration the litter received throughout the lactation period, the 72 mg. for the entire litter of six was evidently sufficient for weaning. So far, out of about thirty litters studied, this is the minimum curative dosage we found. Food consumption records show that the mother of that litter consumed only 12 gm. during the 24 hour period subsequent to the cod liver oil therapy of the litter. This is considerably less than was consumed during any of the 3 preceding days when the litter showed maintenance. Subsequent consumption was, however, considerably more. While we have not found food consumption records of any material help in interpreting all of our data in connection with our studies of the fat-soluble vitamin requirements

for lactation, we have found such records indispensable in analyzing our findings relating to the vitamin B requirements for lactation.

Table III shows clearly that the litter of Female 4744 showed no

TABLE III.

Lactation Record of Females 4743 and 4744 (Second Generation) Transferred from Stock Diet 2 on Date of Birth of First Litters to Fat-Soluble Vitamin-Deficient Ration 1025.

Date.	Female 4743.*	Litter.	Food consumed during previous 24 hrs.	Female 4744.*	Litter.	Food consumed during previous 24 hrs.
June, 1927	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
30	217					
July						
1	182	28(6)†				
2	178	29	10	234		
3	185	33	22	201	32(6)†	
4	186	36	21	206	35	19
5	188	40	25	207	39	25
6	190	43	21	213	43	27
7	192	50	15	215	52	19
8	194	57	28	214	61	36
9	192	63	24	217	70	27
10	200	67	21	222	76	23
11	196	77	31	220	90	34
12	202	80	25	219	98	22
13	205	90	25	212	108	18
14	203	97	23	211	115	16
15	200	108	32	202	120	17
16	201	98(5)†‡	28	192	125	10
17	202	106	22	188	123	5
18	178	110§	26	180	123§	14
19	203	116	31	173	125‖	8
20	206	123	27	170	124‖	6
21	202	130¶	42	175	126**	19
22	211	138	32	184	139††	31
23	193	140	21	180	147¶	24
24	208	145	28	190	157	42
25	197	151	34	192	179	49
26	200	154	21	200	196	47
27	192	175	27	200	211	44
28	200	182	33	205	215	47
29	198	191	36	202	222	36

TABLE III—*Concluded.*

Date.	Female 4743.*	Litter.	Food consumed during previous 24 hrs.	Female 4744.*	Litter.	Food consumed during previous 24 hrs.
June, 1927	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
30	196	200‡‡	41	204	227	40
Aug. 1				202	241‡‡	45

* Females 4743 and 4744 had litters of eight and six respectively and were given six each to rear.

† Figures in parentheses represent number of young left on date indicated.

‡ One young accidentally killed in the cage, leaving five in the litter.

§ Young opened their eyes.

‖ 0.01 cc. of cod liver oil to each young.

¶ Young eating.

** 0.02 cc. of cod liver oil to each young.

†† Discontinued administration of cod liver oil.

‡‡ Young weaned.

response after two daily administrations of 0.01 cc. of cod liver oil per young, but responded remarkably after one more administration of 0.02 cc. per young; in other words, 0.04 cc. or approximately 37 mg. of cod liver oil per young was sufficient for weaning when administered at the critical stage in lactation when the vitamin reserves in the mother were at the point of depletion.

We feel that we have avoided early infant mortality of the litter of Female 4745 (Table IV) by starting with cod liver oil therapy for the young on the 3rd day of lactation, the litter having shown an absolute maintenance curve for 72 hours, and the response not being due to the mother's increase in food consumption. We calculated from the weight of drops and the single 0.02 cc. administration that each young required a minimum of 54 mg. of cod liver oil for weaning.

Table I shows clearly that the cod liver oil therapy for the young resulted not only in remarkable growth of the litters, but at the same time in marked growth of the mothers. On June 21, the 3rd day after cod liver oil therapy was initiated, we observed that the mothers were lapping traces of cod liver oil from the mouths of their young. After each administration of cod liver oil, droplets unavoidably collect around the mouths of the

TABLE IV.

Lactation Record of Females 4745 and 4746 (Second Generation) Transferred from Stock Diet 2 on Date of Birth of First Litters to Fat-Soluble Vitamin-Deficient Diet 1025.

Date.	Female 4745.*	Litter.	Food consumed during previous 24 hrs.	Female 4746.*	Litter.	Food consumed during previous 24 hrs.
July, 1927	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
5	272			260		
6	208	31		204	32	
7	212	32	13	196	36	12
8	210	31	18	200	42	22
9	213	31†	20	201	45	20
10	215	34‡	13	203	49	13
11	215	37	21	201	55	20
12	222	41†	19	198	61	16
13	221	47†	26	200	72	26
14	225	52‡	20	199	78	21
15	222	59	24	202	88	28
16	228	64	26	206	99	29
17	232	72	20	205	108	22
18	225	83	29	202	117	32
19	229	94	27	204	127	26
20	228	102	26	205	136	22
21	228	110	28	204	143	28
22	225	119	25	206	155	36
23	223	122	18	205	158	24
24	222	132§	20	206	164§	30
25	219	141	18	206	172	56
26	224	147	27	207	181	26
27	214	150	27	202	189	34
28	219	157	20	206	198	37
29	218	168	29	205	202	30
30	212	175	25	206	207	36
31	204	178	24	198	203	26
Aug.						
1	200	180	22	200	206	23
2	200	182	19	205	212	31
3	198	183‖	36	202	215	38
4	204	191‡	32	204	220	38
5	195	214	47	206	219	24
6	203	232	20	206	233	51
7	210	250¶	57	208	244	46

* Females 4745 and 4746 had litters of ten and eight respectively and were given six each to rear.

† 1 small drop (12 mg.) of cod liver oil to each young.

‡ Discontinued administration of cod liver oil.

§ Young eating.

‖ 0.02 cc. of cod liver oil to each young (third administration).

¶ Young weaned.

baby rats. We, therefore, immediately resorted to the practice of placing the mothers in compartments separate from the litters for at least 1 hour after the cod liver oil therapy. It is possible, then, that the mothers received in such manner enough vitamin A and D to make the gains in weight indicated in Table I. Later, instead of separating mothers from young we made it a rule after cod liver oil administrations to wipe the mouths of the young thoroughly with a piece of cheese-cloth. This technique was performed with litters of Females 4733, 4744, and 4745.

Six small drops of cod liver oil administered to Female 4734 (Table II) early in lactation prevented infant mortality of the remaining litter of five.

It seems from studies reported in this communication, judging from minimum requirements of nursing baby rats, that we are forcing too much cod liver oil on young infants, the excess of which is supplying calories rather than needed vitamins. It is a problem that can only be solved, however, by correlating accurate data obtained on small experimental animals with extensive observations by clinicians in pediatric hospitals.

The arguments advanced in previous publications (1) for the possible existence of a specific lactation-promoting factor associated with the antisterility complex, vitamin E, are as follows:

1. The addition of sufficient amounts of wheat oil or small amounts of unsaponifiable matter therefrom to a skimmed milk powder sterility-producing diet, containing an abundance of vitamins A and D, resulted not only in fertility but also in normal rearing of young.

2. The reduction of the concentration of wheat oil from 3 to 1 per cent in the ration still allowed enough of the antisterility factor for fertility, but resulted in considerable infant mortality during the nursing period.

3. The reduction of the concentration of unsaponifiable matter from wheat oil from 0.1 per cent to 0.05 per cent showed no injurious effect on fertility, but considerable impairment in lactation.

4. When cod liver oil was removed from the skimmed milk powder sterility-producing diet and administered separately from the ration, while such procedure was followed at times but not regularly in the pregnant females carrying their litters to term, the percentage of infant mortality was 100 per cent. When the

cod liver oil administered separately from the ration to each female per day carried as little as 4 mg. of a highly concentrated preparation of unsaponifiable matter (practically free from sitosterol) from wheat oil, a lactation efficiency index of 70 per cent was obtained. When the unsaponifiable matter from the wheat oil carried by the cod liver oil was reduced, so that the lactating rats received only 2 mg. per day, considerably greater infant mortality was encountered.

5. From earlier experiments it seemed (4) that a differentiation between the antisterility and lactation-promoting factors of wheat oil could be obtained through studies of oxidation, but later investigations have shown (6) that this is not possible, since oxidation renders wheat oil toxic, thus producing a decidedly injurious effect on growth.

6. When females with their litters were transferred during the latter part of pregnancy from Stock Diet 1 to the skimmed milk powder reproduction-deficient diet containing cod liver oil, they gave birth to normal litters and reared their young up to the 15th to the 17th day of lactation, at which period the young reached a maintenance curve. If at this critical stage, when the young were still in reparable condition, an ethereal extract of wheat embryo was administered to the mothers, we were able to save a number of litters. Such data were interpreted to signify that the lactating mothers depleted their reserves of the lactation-promoting factor of vitamin E, and that the young responded when the ethereal extract of wheat germ was supplied to the mothers. Whatever may be the factors involved in the responses obtained in rearing young on the skimmed milk powder diets, we certainly cannot conclude from the transfer experiments from our stock diets to the purified synthetic fat-soluble vitamin-deficient rations reported in this paper that there is any basis for postulating the existence of a specific fat-soluble galactagogic vitamin essential for rearing of the young, other than vitamins A and D. At least, the results obtained on storage through transfer experiments to the milk diets must still be considered an unsolved dietary problem worthy of further research, and we are continuing the investigation.

The lactation-promoting properties of wheat oil have been recently confirmed by Clayton (8) who states: "The presence of

wheat germ oil in the fat-containing rations favored reproduction and also lactation, especially in skim milk powder rations." Whether or not vitamin E should be divided into two specific factors, one essential for fertility and one for lactation, must, however, still await more convincing evidence.

SUMMARY.

1. When females with their litters are transferred from a stock cereal diet containing 1 per cent cod liver oil (supplemented by a liberal supply of fresh milk daily) to a purified synthetic fat-soluble vitamin-deficient diet, there is enough storage of fat-soluble vitamins for normal lactation.

2. When females with their litters are transferred from a stock whole wheat (two-thirds) and whole milk powder (one-third) diet, containing 9 per cent milk fat, to a purified synthetic fat-soluble vitamin-deficient ration, depletion of reserves of fat-soluble vitamins is encountered in the second generation.

3. A method of studying the cod liver oil requirements of nursing young has been outlined.

4. From the detailed quantitative experiments of depletion of reserves of fat-soluble vitamins during the nursing period, no evidence could be obtained of the existence of a specific vitamin essential for lactation, other than vitamins A and D.

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DIETARY REQUIREMENTS FOR FERTILITY AND LACTATION.

XIV. A QUANTITATIVE BIOLOGICAL METHOD FOR THE STUDY OF VITAMIN B REQUIREMENTS FOR LACTATION.*

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In a previous communication (1) it was demonstrated that the vitamin B requirements for normal lactation are at least 3 times those required for optimum growth. This investigation was conducted in order to secure more quantitative data on the vitamin B requirements for milk secretion, and also with the purpose of possibly solving the question why the mammary gland requires such large proportions of a vitamin for the physiological function of lactation compared with that for optimum growth of the adult.

In order to place the experimentation on an absolute quantitative basis, it was decided to abandon entirely the group method of feeding and place each animal in an individual cage on raised screens, so that there would be no access to excreta, to secure accurate records of food consumption, and to administer the vitamin daily separate from the ration; in other words, the Osborne and Mendel type of experimentation was adopted, together with the Steenbock type of false screen bottoms (2). It was anticipated that greater progress in this problem could be achieved by reducing the length of the experimental period to the minimum and limiting all detailed observations to the nursing period. For that reason,

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TABLE I.

Lactation Record of Females 4416 and 4417 (First Litters) Transferred from Stock Diet 1 on Date of Birth of Their Litters to Vitamin B-Deficient Diet 988.

Potency of Harris Yeast as Source of Vitamin B for Lactation.

Composition of Ration 988: casein (purified) 20.0, agar-agar 2.0, McCollum's Salt Mixture 185 4.0, butter fat 10.0, dextrin 64.0

Date.	Female 4416.*	Litter.	Food consumed during previous 24 hrs.	Female 4417.*	Litter.	Food consumed during previous 24 hrs.
Nov., 1928	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
21	227	32				
22	210	35	11	192	28	
23	212	38	17	187	31	14
24	209	44	17	184	35	14
25	207	49	13	182	39	11
26	202	58	17	177	47	14
27	192	63	11	173	52	14
28	192	67	16	167	62	16
29	187	77	14	166	69	12
30	177	75(5)†	13	162	72	14
Dec.						
1	173	79	12	153	77	11
2	171‡	82	16	153‡	77	11
3	179	86	14	166	76	16
4	183	85	16	162	78	17
5	183§	87	17	167§	82	18
6	187	92	17	172	82	17
7	192	93	17	176	86	22
8	198	87	19	175	92	17
9	198	82	18	173	98	18
10	207	43(3)†‖	21	173	105	19
11	202	33(2)†¶	16	169**	105	19
12				177	109	21
13				182	113	24
14				177	118	21
15				173	127	19
16				177	130	23
17				177	136	26
18				181	145	28
19				178	152	23
20				184	156	24
21				184	163	23
22				183	171	26
23				183	176	23

TABLE I—*Concluded.*

Date.	Female 4416.*	Litter.	Food consumed during previous 24 hrs.	Female 4417.*	Litter.	Food consumed during previous 24 hrs.
Dec., 1926	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
24				183	183	24
25				186	186	25
26				180	191	25
27				187	199	25
28				188	218	34
29				196	223	30
30				193	223	32
31				193	241††	28

* Females 4416 and 4417 had litters of eleven each and were allowed six young each to rear.

† Figures in parentheses represent number of young left on date indicated.

‡ Administered 500 mg. of Harris yeast (triturated with 5 drops of cod liver oil) daily to mother separately from ration.

§ Increased yeast (triturated with 5 drops of cod liver oil) to 750 mg. daily to mother.

‖ Two young were found dead on the morning of December 10. Necropsy: Both young showed marked congestion at juncture of occipital and parietal bones. The stomach of one of the young was full of curd, that of the other was empty.

¶ One of the remaining two young had paralysis and muscle chills at 3 p.m., December 11; chloroformed. Necropsy: Stomachs of both young full of curd; marked congestion at juncture of occipital and parietal bones.

** Further increased yeast (triturated with 5 drops of cod liver oil) to 1500 mg. daily to mother.

†† Litter weaned.

the technique employed was as follows: Mothers with their litters, reduced to six in number, were transferred from our colony Stock Diet 1 (3) to a vitamin B-deficient ration, consisting of casein[1] (purified) 20.0, agar-agar 2.0, McCollum's Salt Mixture 185 (4) 4.0, butter fat 10.0, and dextrin 64.0. It was found that lactating females transferred from the diet of our regular Stock Colony 1 to such a dietary régime rear their young for 1 to 2 weeks, depending on the amount of storage, during which period the mothers lose from 20 to 30 per cent of their body weights in their attempts to prolong lactation. The young then reach a maintenance

[1] The casein was purified by washing for a week with dilute acetic acid.

TABLE II.

Lactation Record of Females 4496 and 4497 (First Litters) Transferred from Stock Diet 1 on Date of Birth of Their Litters to Vitamin B-Deficient Diet 1009.

Potency of Harris Yeast as Source of Vitamin B for Lactation.

Composition of Ration 1009: casein (purified) 20.0, agar-agar 2.0, McCollum's Salt Mixture 185 4.0, butter fat 5.0, dextrin 69.0.

Date.	Female 4496.*	Litter.	Food consumed during previous 24 hrs.	Female 4497.*	Litter.	Food consumed during previous 24 hrs.
Feb., 1927	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
21	244			297		
22	180	31		247	28	
23	179	35	9	226	29	1
24	172	40	10	220	32	5
25	170	45	11	217	37	10
26	168	51	12	207	42	6
27	166	57	16	200	46	6
28	163	60	13	194	50	18
Mar.						
1	153	66	10	189	55	10
2	152	71	12	185	66	10
3	149	75	15	173	66	8
4, 9 a.m.	143†	77	10	172	71	9
10 p.m.	153	80	12‡			
5, 9 a.m.	151	83	18	172	77	14
10 p.m.	161	87	13§			
6	155	93	18	163	83	10
7	157	102	23	159†	85	13
8	160	109	20	168	91	17
9	167	116	29	173	98	22
10	170	124‖	23	174	105	24
11	169	132	26	173	112	20
12	169	141	24	178	119	23
13	170	146	27	178	123	23
14	166	154	25	185	129	27
15	175¶	157	21	182	134	26
16	169	166**	25	173¶	133**	20
17	170	172	31	191	148	29
18	170	181	20	197	154	24
19	176	194	31	197	155	29
20	176	198	35	196	156	28
21	178	206	33	200	160	35
22	177	214	35	205	166	36

TABLE II—*Concluded.*

Date.	Female 4496.*	Litter.	Food consumed during previous 24 hrs.	Female 4497.*	Litter.	Food consumed during previous 24 hrs.
Mar., 1927	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
23	179	223	28	206	173	27
24	177	232	33	206	182	33
25	178	242††	33	214	190	34
26				206	205	43
27				211	214	34
28				208	232	36
29				210	240††	36

* Females 4496 and 4497 had litters of eleven and twelve young respectively and were allowed six each to rear.

† 1000 mg. of Harris yeast administered to mother daily separately from the ration.

‡ Food consumption between 9 a.m. and 10 p.m. on March 4. Between 10 p.m. on March 4 and 9 a.m. on March 5, 6 gm. of additional food were consumed.

§ Food consumption between 9 a.m. and 10 p.m. on March 5.

‖ Young opened their eyes.

¶ Increased yeast daily dosage to mother to 1500 mg.

** Young first observed eating.

†† Litter weaned.

curve showing depletion of the vitamin reserves of the mother. At that critical point the curative method was employed by administering to the mothers yeast (weighed accurately to the mg.) separately from the ration in Petri dishes. Mothers and young were weighed daily, and daily records were kept of food consumption.[2] The first experiments were begun with Harris yeast.[3]

[2] In several instances, because of pressure of other work, we were compelled to delete the daily records of food consumption until the period of maintenance of the litters. In a few other cases we were obliged to take food consumption records for 48 and 72 hour periods instead of for 24 hour periods. All our experimental animals receive liberal supplies of distilled water daily, and small amounts of iodine in the water once a week.

[3] Two brands of Harris yeast were employed in this investigation: "Harris yeast" refers to the product employed by us for several years, which is a dehydrated brewers' yeast extract referred to in the text; "Brewers' Yeast-Harris (Medicinal)" according to the manufacturers is a dehydrated tested brewers' yeast. Detailed data are presented only on the "Harris yeast."

For several years the author had used this product as a satisfactory supplement for purposes of lactation to a skimmed milk powder basal, reproduction-deficient diet. It became necessary now, however, to know definitely the biological value of this brewers' yeast when examined under a dietary régime composed of purified food substances. On the synthetic ration described above it became apparent that while 300 mg. of this Harris yeast per rat per day was sufficient for satisfactory growth for a period of 4 months, it was necessary to increase the daily dosage to 500 mg. per animal in order to secure optimum growth for a period of 7 months. Such findings were rather surprising, in view of the manufacturers' claim that this brewers' yeast is a concentrated extract prepared by the method of Osborne and Wakeman (5) the original product of which Osborne and Mendel (6) found very potent in daily allowances of 15 to 20 mg. per 100 gm. of body weight. Knowing, however, that 500 mg. are the daily animal dosage required for optimum growth of the adult, we were now ready to determine accurately the minimum daily requirement for lactation. When the first experiments were initiated, we employed 10 per cent butter fat in the purified ration, and, in order to insure enough of the antirachitic factor, the Harris yeast was triturated daily with 5 drops of cod liver oil. Later, when it became quite clear that there was enough storage of all fat-soluble vitamins from the previous dietary (7), the butter fat in the rations was reduced to 5 per cent and the drops of cod liver oil removed from the yeast (see Tables I and II). The results of the experiments are shown in detail in Tables I to IV.

DISCUSSION.

Table II shows that 1000 mg. of Harris yeast daily to each lactating rat, twice the amount required for optimum growth of the adult, as a source of vitamin B, are not adequate to meet the demands for lactation, and that it is necessary to supply as much as 1500 mg. daily to each nursing female to avoid infant mortality. Applying the same technique of administering 1500 mg. of other types of yeast to the nursing mother at the critical point of depletion of vitamin B reserves, Brewers' Yeast-Harris (Medicinal) was found considerably inferior to the Harris yeast (concentrated ex-

tract), as indicated by the lactation efficiency index; and Schlitz brewers' yeast[4] more potent than the Harris medicinal yeast. The Schlitz yeast showed a lactation efficiency index of 75 per cent while the Brewers' Yeast-Harris (Medicinal) showed an efficiency index of only 50 per cent. Having found considerable differences in the biological potency of the three types of yeast assayed by the biological method herein described, we were then ready to test concentrated preparations from yeast (prepared by another method) as a source of vitamin B for lactation.

In 1921 the Research Laboratories of Eli Lilly and Company adsorbed large amounts of a bakers' yeast on fullers' earth, and fortunately had an appreciable stock of that adsorbed product on hand. Mr. Elmer H. Stuart, Research Chemist of the Lilly laboratories, cooperating in this investigation, released about 15 pounds of the adsorbed vitamin product by a modified procedure of the recent Seidell method (8), employing sodium hydroxide for the extraction and then neutralizing the alkali with calculated amounts of sulfuric acid. All preparations were dried in vacuum pans. Yeast Concentrate P-19618 represents the product released with sodium hydroxide, and Yeast Concentrate P-19619 washings of the sodium sulfate crystals (7).

In this paper detailed data on Yeast Concentrate P-19618, which is the more concentrated product, are submitted. Table III shows clearly that while a dosage of 300 mg. daily brought an appreciable response both in the lactating mother and nursing young, it was necessary to increase the daily dosage to the mother to 500 mg. in order to supply enough vitamin B for the successful weaning of the litters; in other words, the Lilly concentrated yeast product, No. P-19618, was found 3 times as potent as the Harris yeast.

The author has prepared in his laboratory the crude picrate from Yeast Concentrate P-19618 which gave excellent responses in daily dosages of 5 mg., or 2.5 mg. of the vitamin, resulting in gains of 14 gm. in 6 days during postlactation periods when administered to females whose vitamin B reserves had been entirely

[4] The Schlitz brewers' yeast is a dehydrated product prepared about 7 years ago by drying fresh brewers' yeast at a temperature of 100°. This product was kindly furnished the author by Mr. W. H. Rhode, Chief Chemist of the Schlitz Beverage Company, Milwaukee.

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TABLE III.

Lactation Record of Females 4623 (First Litter) and 4624 (Second Litter) Transferred from Stock Diet 1 on Date of Birth of Litters to Vitamin B-Deficient Diet 1009.

Potency of Lilly Yeast Concentrate P-19618 as Source of Vitamin B for Lactation.*

Date.	Female 4623.†	Litter.	Food consumed during previous 24 hrs.	Female 4624.†	Litter.	Food consumed during previous 24 hrs.
May, 1927	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
13	222			340		
14	180	32		258	33	
15	164	34	0	256	39	8
16	162	37	12	252	48	12
17	155	37	9	244	52	17
18	156	42	5	236	60	9
20	150	52		225	77	
22	145	61		211	88	
23	142	66	49‡	208	95	64‡
24	136	72	5	205	102	10
25	134§	75	10	197§	105	10
26	142	80	18	205	113	22
27	143‖	79	20	206	123	24
28	144	94	18	210	130	12
29	146	98¶	20	208	140¶	22
30	151	105	23	207	144	22
31	150	114	20	203	151	21
June						
1	147	117	20	207‖	153	27
2	152	123	24	206	160	27
3	150	131	22	203	171	31
4	154	135	22	208	180	29
5	158	144**		218	196**	
6	159	157	65††	215	213	81††
7	157	162	22	208	222	33
8	163	166	23	210	232	43
9	167	178	30	210	235	29
10	168	191	34	207	243‡‡	37
11	169	197	33			
12	173	206	31			

TABLE III—*Concluded.*

Date.	Female 4623.†	Litter.	Food consumed during previous 24 hrs.	Female 4624.†	Litter.	Food consumed during previous 24 hrs.
June, 1927	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
13	171	220	40			
14	164	226	28			
15	171	231	33			
16	178	232	31			
17	172	236§§	26			
18	175	252‡‡	42			

* For method of preparation, see text.

† Females 4623 and 4624 had litters of nine and eleven young respectively and were given six each to rear.

‡ Food consumption record for 48 hours.

§ 300 mg. of Lilly Yeast Concentrate P-19618 to mothers daily.

‖ Increased yeast dosage to 500 mg. to mother daily.

¶ Young opened their eyes.

** Young eating.

†† Food consumed during a 72 hour period.

‡‡ Litter weaned.

§§ Yeast concentrate administered to litter instead of to mother.

depleted. The responses were noted not only in growth, but also in food consumption (9).

The marked increases in food consumption following the administration of yeast or yeast concentrates to the lactating females is apparent in all the experiments in this investigation. The unusual gains in weight of the nursing mothers in as short an interval as 24 hours need some comment. For instance, Female 4497 (Table II, March 17) gained 18 gm. in 24 hours subsequent to the administration of 1000 mg. of Harris yeast. Females 4625 and 4626 (Table IV, May 26) gained 12 gm. each in 24 hours subsequent to an administration of 500 mg. of the Yeast Concentrate P-19618. It is very doubtful whether such rapid gains in body weight could be ascribed to the building of muscle tissue, to storage of glycogen in the liver, or to synthesis of adipose tissue. It is more probable that in vitamin B starvation we have a dehydration of tissue and that the rapid gains in body weight in the adult are nothing more than a replenishment of essential metabolic water.

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In the communication following it will be shown that the large requirements of vitamin B for successful lactation are due to the inability of the lactating mother to secrete that vitamin quantitatively in the milk.

TABLE IV.

Lactation Record of Females 4625 and 4626 (First Litters) Transferred from Stock Diet 1 on Date of Birth of Litters to Vitamin B-Deficient Ration 1009.

Potency of Lilly Yeast Concentrate P-19618 as Source of Vitamin B for Lactation.*

Date.	Female 4625.†	Litter.	Food consumed during previous 24 hrs.	Female 4626.†	Litter.	Food consumed during previous 24 hrs.
May, 1927	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
14	296			292		
15	220	30		215	36	
16	220	33	7	212	43	10
17	220	33	13	207	44	14
18	222	40	8	207	51	9
20	212	50		204	65	
21	205	55		194	72	
22	204	60		190	78	
23	198	66	59‡	187	84	73‡
24	192	73	5	182	92	9
25	182§	75	7	180§	93	11
26	194	84	21	192	102	22
27	195	90	20	190	108	22
28	200	98	25	192	114	20
29	196	106	25	194	120	30
30	201	115	26	196	127‖	25
31	202	125	22	195	137	19
June						
1	200	132	25	194	145	22
2	204	140	25	196	152¶	22
3	204	146	26	199	160	37
4	203	156	29	198	170	30
5	204	165¶		200	178	
6	204	175	60**	201	191	65**
7	205	187	31	200	195	38
8	205	195	34	205	201	35
9	210	213	33	206	211	32
10	208	223	32	210	222	40

TABLE IV—*Concluded.*

Date.	Female 4625.†	Litter.	Food consumed during previous 24 hrs.	Female 4626.†	Litter.	Food consumed during previous 24 hrs.
June, 1927	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
11	211	227	34	207	227	34
12	209	237	33	212	228	32
13	210	248††	34	214	233	34
14				215	244††	39

* For method of preparation, see text.

† Females 4625 and 4626 had litters of eight each and were allowed six each to rear.

‡ Food consumption record for a 96 hour period.

§ 500 mg. of Lilly Yeast Concentrate P-19618 to mothers daily.

‖ Young opened their eyes.

¶ Young eating.

** Food consumption record of a 48 hour period.

†† Young weaned.

SUMMARY.

1. A quantitative biological method for the study of vitamin B requirements for normal lactation is described.

2. By such a method it became apparent that yeasts from various sources vary considerably in their biological value as a source of vitamin B for lactation.

3. It was found that a concentrated preparation from yeast, prepared by releasing vitamin B of the yeast adsorbed on fullers' earth with NaOH, is 3 times as potent for normal lactation as the Harris yeast.

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DIETARY REQUIREMENTS FOR FERTILITY AND LACTATION.

XV. THE INEFFICIENCY OF THE LACTATING MOTHER (MUS NORVEGICUS ALBINUS) IN SECRETING VITAMIN B IN THE MILK, AND THE RELATION OF THIS PHENOMENON TO INFANT MORTALITY (DETAILED REPORT).*†

By BARNETT SURE.

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(Received for publication, October 31, 1927.)

After finding the Harris yeast prohibitive in cost, calculated in terms of its potency, for the extensive needs of our fat-soluble vitamin investigations, we abandoned the use of that product and began to employ dehydrated yeast furnished by the Northwestern Yeast Company. This yeast we later learned is powdered Yeast Foam tablets.

In assaying the Northwestern yeast as a source of vitamin B for lactation by the biological method described in the previous communication (3) it became apparent that, by administering this yeast to the nursing mothers in graduated amounts at points of maintenance of the young, rearing and weaning of the young could be accomplished only by a considerable prolongation of the nursing period, and in some cases the percentage of infant mortality was

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† Preliminary reports of this investigation have appeared in recent issues of the *Journal of the American Medical Association* (1) and of *Science* (2). Only part of our detailed data are submitted in this paper.

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TABLE I.

Lactation Record of Females 4643 (First Litter) and 4644 (Second Litter) Transferred from Stock Diet 1 on Date of Birth of Litters to Ration 1009.

Potency of Northwestern Yeast as Source of Vitamin B for Lactation.

Composition of Ration 1009: casein (purified) 20.0, agar-agar 2.0, McCollum's Salt Mixture 185 (4) 4.0, butter fat 5.0, dextrin 69.0.

Date.	Female 4643.*	Litter.	Food consumed during previous 24 hrs.	Female 4644.*	Litter.	Food consumed during previous 24 hrs.
May, 1927	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
20	244			340		
21	198	35		278	35	
23	193	47		267	40	
25	182	60		258	52	
28	172	80		250	77	
30	165	91		243	93	
31	159	95	12	232	98	13
June						
1	157	99	6	230	103	10
2	153	100	11	223	110	15
3	149†	100	9	215†	115	13
4	153	101	14	209	117	10
5	153	109		210	120	
6	152	114	35‡	205†	120	29‡
7	149	114	9	209	121	13
8	149	118	24	207	123	23
9	147	121	8	206	127	13
10	144	123	16	207	132	19
11	144	124	14	208	134	19
12	143	124	14	209	139	21
13	143	122	16	210	141	22
14	142	115	12	210	140	23
15	152	118	19	214	140	24
16	147	121	19	214	141	20
17	147§	123	19	219§	142	23
18	153	129	23	229	142	25
19	157	135		234	142	
20	157	146	50‡	237	141	58‡
21	165	154	32	242	138‖	29
22	166	157	30	243	98(4)¶	26
23	160	164	24	251	99	24
24	173	170	39	253	96	24
25	174	180	38	253	72(3)	

TABLE I—*Concluded.*

Date.	Female 4643.*	Litter.	Food consumed during previous 24 hrs.	Female 4644.*	Litter.	Food consumed during previous 24 hrs.
June, 1927	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
26	176	190		254	72(3)**	
27	178	205	70‡			
28	178	213	36			
29	178	214	32			
30	183	221	35			
July						
1	185	230	33			
2	188	236	40			
3	188	241††	31			

* Females 4643 and 4644 had litters of eight and nine young respectively and were allowed six each to rear.

† 500 mg. of Northwestern yeast to the mother daily.

‡ Food consumed during a 48 hour period.

§ Increased yeast dosage to 1500 mg. to mothers daily.

‖ Two young with marked posterior paralysis and muscle chills.

¶ Figures in parentheses represent number of young left on date indicated.

** Experiment discontinued.

†† Litter weaned.

alarming even when the lactating mother received as much as 1500 mg. daily of this yeast. It was discovered, however, that rearing of the young could be expedited and infant mortality at the same time avoided if, at a certain critical stage in lactation when the young were failing, the greater portion of the yeast dosage was allowed to the young instead of to the mother. Tables I to III offer convincing evidence of the inability of the nursing mother to secrete the vitamin B, furnished by the Northwestern yeast, quantitatively in the milk, and show that the mothers store that vitamin in their tissues when the young are dying or struggling for independence.

Female 4643 (Table I) required 16 days to wean its litter after the administration of 1500 mg. daily of Northwestern yeast, and during this period the mother recovered 28 per cent in body weight. Female 4644 failed to rear its litter after the increased daily dosage of 1500 mg. of yeast, and was storing vitamin B in its tissues during

the stage of failure of the litter. During the period the young were failing this mother added 35 gm. of body weight in 9 days. After the litter was reduced to three the experiment was discontinued. In striking contrast, it will be noted, that rearing of the young

TABLE II.

Lactation Record of Females 4651 and 4652 (Second Litters) Transferred from Stock Diet 1 on Date of Birth of Litters to Vitamin B-Deficient Ration 1009.

Shortening of Nursing Period and Reduction of Infant Mortality by Administering Vitamin B to Litters Instead of to Mothers during Later Stages of Lactation.

Date.	Female 4651.*	Litter.	Food consumed during previous 24 hrs.	Female 4652.*	Litter.	Food consumed during previous 24 hrs.
May, 1927	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
22	300					
23	245	31		236		
24	230	35		300	34	
26	219	44		279	47	
28	201	59		272	66	
30	194	70		253	82	
June						
1	185	76		237	97	
2	172	81	4	233	104	14
3	168	85	11	225	110	12
4	166†	87	9	219†	114	8
5	174	93	15	216	120	17
6	171	97	17	222	128	18
7	167	102	13	217	133	16
8	169	109	23	215	140	26
9	165	114	10	212	142	12
10	167	120	20	211	144	19
11	166	122	10	211	148	20
12	165	129	20	208	146	18
13	164	132	19	210	147	22
14	165	131	17	206	149	18
15	164	131	11	206	150	19
16	160	130	16	210	149	23
17	163‡	130	20	210‡	151	21
18	169	146	37	227	154	34
19	181	148§	30	234	160§	30
20	186	153	30	232	166	31

TABLE II—*Concluded.*

Date.	Female 4651.*	Litter.	Food consumed during previous 24 hrs.	Female 4652.*	Litter.	Food consumed during previous 24 hrs.
June, 1927	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
21	186‖	159	38	234‖	174	33
22	176	172	34	228	192	40
23	170	190	32	227	214	39
24	167	210	42	218	221	37
25	166	224	41	216	240¶	48
26	159	237¶				

* Females 4651 and 4652 had eleven and fourteen young respectively and were allowed six each to rear.

† 500 mg. of Northwestern yeast to mothers daily.

‡ Increased yeast to 1500 mg. to mothers daily.

§ Young eating.

‖ Yeast removed from mothers and same dosage administered to litters daily.

¶ Litter weaned.

was considerably expedited and infant mortality was entirely overcome in the case of the litters of Females 4651, 4652 (Table II), and Female 4656 (Table III) through the simple expedient of removing during the later stage of lactation the 1500 mg. daily yeast dosage from the mothers and administering it to the litters. Litters of Females 4652 and 4651 were weaned in 4 and 5 days respectively after that change was made. Litter of Female 4656 was weaned in 4 days by this technique. Note the great infant mortality of litter of Female 4655 even after an increased daily dosage of 1500 mg. of yeast was given to the mother.

Having already determined that the minimum daily dosage of Yeast Concentrate P-19618 is 500 mg. when all the vitamin B is expected to be transferred to the young through the mother's milk, we next proceeded to investigate what the requirements of such vitamin concentrate would be per nursing young per day, if the same were distributed between mother and young according to their needs. The lactation record of Female 4695 (Table IV) serves as an excellent illustration.

On June 14, at the first maintenance point of the litter, a daily therapeutic dosage of 100 mg. was allowed the mother. In 3 days

TABLE III.

Lactation Record of Females 4655 and 4656 (First Litters) Transferred from Stock Diet 1 on Date of Birth of Litters to Vitamin B-Deficient Ration 1009.

Shortening of Nursing Period and Reduction of Infant Mortality by Administering Vitamin B to Litters Instead of to Mothers during Later Stages of Lactation.

Date.	Female 4655.*	Litter.	Food consumed during previous 24 hrs.	Female 4656.*	Litter.	Food consumed during previous 24 hrs.
May, 1927	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
24	302			260		
25	240	40(6)†		198	34(6)†	
26	240	47		190	38	
28	234	68		188	54	
30	226	82		178	67	
June						
1	222	101		168	77	
2	215	110		168	82	
3	210	117	17	160	88	11
4	204	124	10	155	91	6
5	197	125		156	96	
6	191‡	124	27§	150‡	98	22§
7	200	129	13	151	101	13
8	195	135	22	153	108	22
9	191‖	135	12	153‖	110	10
10	200	138	25	161	117	21
11	199	143	33	161	122	16
12	200	148	25	161	130	19
13	198	153	27	165	136	26
14	204¶	154	27	163¶	140	21
15	206	76(3)†	32	172	147	28
16	216	26(1)†	21	168	152	24
17				173	156	28
18				179	160	31
19				180	166	30
20				184	174	31
21				181	175**	26
22				175	196	36
23				170	216	32
24				167	233	32
25				163	249††	38

* Females 4655 and 4656 had litters of seven and ten young respectively and were allowed six each to rear.

† Figures in parentheses represent number of young left on date indicated.

‡ 500 mg. of Northwestern yeast to mother daily.

§ Food consumption record of a 48 hour period.

‖ Increased yeast dosage to mothers to 1000 mg. daily.

¶ Increased yeast dosage to mothers to 1500 mg. daily.

** Yeast dosage administered to litter instead of to mother.

†† Litter weaned.

TABLE IV.

Lactation Record of Female 4695 (First Litter) Transferred from Stock Diet 1 on Date of Birth of Litter to Ration 1009.*

Distribution of Vitamin B between Mother and Young According to Need.

Date.	Female 4695.	Litter.	Food consumed during previous 24 hrs.	Date.	Female 4695.	Litter.	Food consumed during previous 24 hrs.
June, 1927	*gm.*	*gm.*	*gm.*	*June, 1927*	*gm.*	*gm.*	*gm.*
1	254			25	160	128	26
2	208	29		26	160	138	18
3	195	35		27	158	146††	25
6	184	49		28	160	150	27
8	174	55		29	160	154	26
10	170	71		30	158‡‡	157§§	
11	165	76		*July*			
13	153	83		1	165	162|||	27
14	148†	84	13	2	161	165	22
15	160	85	12	3	166	174	27
16	159	91	13	4	158	181	32
17	165‡	93	19	5	156	188	24
18	159§	93||	16	6	160	199	32
19	163	98	15	7	160¶¶	204	27
20	164	102	16	8	165	212	29
21	166¶	106	20	9	160	226	30
22	162	112	20	10	160	238***	36
23	158	117	20				
24	158**	119	23				

* Gave birth to nine young; allowed six to rear.

† 100 mg. of Lilly Yeast Concentrate P-19618 to mother daily.

‡ 10 mg. of concentrate to mother; 90 mg. to litter daily.

§ 30 mg. to mother; 90 mg. to litter daily.

|| Young opened their eyes.

¶ 150 mg. of concentrate to mother; none to litter.

** 180 mg. to mother; none to litter.

†† Young eating.

‡‡ 60 mg. of concentrate to mother, 120 mg. to litter daily.

§§ The entire litter showed symptoms of posterior paralysis, two out of the six young having developed screaming, running fits. Vitamin therapy to the entire litter was immediately begun, allowing 20 mg. to each young of Lilly Yeast Concentrate P-19618. Spasms ceased in the two young immediately after a few drops of the vitamin in aqueous solution were administered with a medicine dropper.

||| No signs of paralysis or spasms the next morning. Young actively playing and observed frequently nursing.

¶¶ 30 mg. of concentrate to mother; 150 mg. to litter daily.

*** Litter weaned.

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the mother gained 17 gm. and the litter only 9 gm. On June 17 the 100 mg. allowance was distributed between mother and young, the mother being given only 10 mg. and the litter 90 mg. The vitamin was administered to the young in aqueous solution with a medicine dropper. Since the mother lost 6 gm. during the subsequent 24 hours and also reduced its food consumption, it was quite apparent that the mother needed more vitamin B for its own welfare. On June 18 the mother's daily dosage was, then, increased to 30 mg., leaving the same daily allowance of 90 mg. to the litter of six, or 15 mg. to each young. During the subsequent 3 days the mother gained 7 gm. and at the same time showed a definite increase in food consumption; the litter gained 13 gm. On account of the small daily gains of the litter, however, it was decided to abandon vitamin therapy to the nursing young and determine what the mother could accomplish in lactation with a daily administration of 150 mg. The litter responded appreciably during the first 48 hours, but gained only 2 gm. during the subsequent 24 hour period, the mother having lost 8 gm. during this 72 hour experimental period. On June 24 the daily dosage to the mother was increased to 180 mg. The litter responded most markedly the first 3 days, gaining 27 gm., but the following 3 days it gained only 11 gm. The mother showed a maintenance curve. On June 30, the 6th day after the mother began to receive the 180 mg. daily dosage, the entire litter showed symptoms of posterior paralysis, two out of the six young having developed screaming, running fits. Vitamin therapy to the entire litter was immediately begun, allowing 120 mg. to the litter (20 mg. to each young) and only 60 mg. to the mother. Spasms ceased in the two young immediately after a few drops of the vitamin in aqueous solution were administered to them with a medicine dropper. No signs of paralysis or spasms were noted in any of the young on the following morning, and they were observed actively playing and frequently nursing during the afternoon following the vitamin therapy. By such distribution of vitamin between mother and young the litter gained 47 gm. during a subsequent 8 day experimental period. The food consumption also increased appreciably, partly because the young were able to partake of some of the mother's ration. Since during the last 8 day period the mother lost no weight, and in fact, gained 2 gm., and

TABLE V.

Lactation Record of Female 4622 (First Litter of Eleven Young, Six Allowed to Be Reared) Transferred from Stock Diet 1 on Date of Birth of Litter to Ration 1009.

Unusual Response to Vitamin B Therapy of Nursing Young at a Stage of Paralysis, Chills, and Screaming, Running Fits.

Date.	Female 4622.	Litter.	Food consumed during previous 24 hrs.	Date.	Female 4622.	Litter.	Food consumed during previous 24 hrs.
May, 1927	*gm.*	*gm.*	*gm.*	*June, 1927*	*gm.*	*gm.*	*gm.*
13	332			1	184‡	121	21
14	268	38		2	196	120	18
15	253	39	7	3	204	108(5)§	23
16	244	40	13	4	195	112	18
17	242	46	15	5	200	110	19
18	236	52	11	6	200	113‖	21
20	231	66	30*	7	196	113	20
22	224	79		8	200	114¶	25
23	216	87	38†	9	201	118	16
24	212	95	12	10	205	133**	34
25	206	101	11	11	222	157	36
26	202	109	15	12	208	163	37
27	193	113	12	13	208	177	44
28	190	117	11	14	210	188	36
29	189	119	17	15	212	201††	37
30	188	118	14				
31	192	123	13				

* Food consumed during a 48 hour period.

† Food consumed during a 72 hour period.

‡ 500 mg. of Yeast Concentrate P-19619 (washings of the sodium sulfate crystals of Yeast Concentrate P-19618, described in text) to mother daily.

§ One young found dead in cage; five out of six left.

‖ Young found in feeding pan.

¶ On the afternoon of June 8, entire litter of five showed paralysis and muscle chills, and three young screaming, running fits. One of the convulsive young jumped out of the attendant's hands three times as it was being held for vitamin therapy. The paralysis extended to the jaws, so the convulsive young could swallow only with great difficulty. Vitamin therapy to the entire litter was immediately begun, allowing 450 mg. of Yeast Concentrate P-19619 to the litter, or 90 mg. to each young, and only 50 mg. to the mother. Such distribution of the vitamin dosage was continued daily. After a few drops of the vitamin solution were forced down the throats of the convulsive young they immediately overcame their spasms and could swallow readily in 5 minutes. The rest of the vitamin dosage was then readily administered with a medicine dropper. After 10 minutes the sick baby rats were observed bunched up in a group quietly resting, and in a few hours nursing. They were seen actively playing at 5 p.m. on the next day, June 9.

** Young observed eating.

†† Litter weaned.

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since the litter gained only 5 gm. between July 6 and 7, in order to expedite rearing of the young 30 mg. were removed from the mother's daily dosage and given to the young; in other words, a daily dosage of only 30 mg. was given to the mother and 150 mg. to the litter of six, or 25 mg. to each young. By this procedure the litter was weaned in the next 3 days, having gained 34 gm. during the last experimental period, and the mother was still able to maintain its own weight on only 30 mg.

It is quite clear, then, that while a daily dosage of 300 mg. of Yeast Concentrate P-19618 is an absolute failure as a source of vitamin B when the nursing mother is expected to secrete the vitamin in the milk (3), 500 mg. being the minimum daily requirement for such physiological function, 180 mg. of the same vitamin B concentrate, distributed between mother and young according to their needs, give absolute success in rearing and weaning of the young, and at the same time provide for maternal welfare. We have calculated from similar results obtained from 50 experiments, in which Yeast Concentrate P-19618 was employed, that the lactating mother wastes at least 60 per cent, if not more, of the daily vitamin B dosage apportioned to it in the metabolism of transfer to the milk.

Table V offers further convincing evidence of the inefficiency of the lactating mother in secreting vitamin B in the milk. When rearing of the young at a certain stage in lactation became a complete failure when the mother (Female 4622) received 500 mg. of Yeast Concentrate P-19619 (our less concentrated vitamin B preparation) it became a complete success when the young received 450 mg. and the mother only 50 mg.

We next proceeded to concentrate further Yeast Concentrate P-19618 by employing a modification of Levene and van der Hoeven's method, consisting of adsorption on silica gel and the release of the vitamin by extraction at various hydrogen ion concentrations (5). At this writing we have weaned litters of young by a distribution of a total of 45 mg. daily between mother and young, the mother receiving a maximum of 5 mg. daily and the nursing young a maximum of 7 mg. each daily.

It was shown in the previous paper (3), that, employing the curative method, we found 1500 mg. of Harris yeast to be the minimum daily dosage as a source of vitamin B for successful

lactation. Using the preventive method by starting daily administrations of 500 mg. of the Harris yeast to the mothers on the

TABLE VI.

Lactation Record of Females 4590 and 4591 (First Litters) Transferred from Stock Diet 1 on Date of Birth of Litters to Ration 1009.

*Accelerated Growth of Nursing Young Following Administration of Liberal Amounts of Potent Concentrated Preparation of Vitamin B.**

Date.	Female 4590.†	Litter.	Food consumed during previous 24 hrs.	Female 4591.†	Litter.	Food consumed during previous 24 hrs.
May, 1927	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
8	216					
9	188‡	35				
10	185	38	12			
11	184	44	11	242‡	30	
12	186	50	18	220	34	6
13	184	55	20	222	34	18
14	184	63	19	228	37	18
15	184	74	20	226	46	15
16	175	80	20	223	50	19
17	174	90	18	224	57	19
18	174	99	20	224	68	22
20	167	110	39§	214	85	30§
22	164	125‖	35§	211	110	46§
24	158	131	37§	205	128	43§
25	160	135	22	198	135	15
26	156	140	12	198	140	19
27	156	142	19	193	147‖	23
28	156	144	16	193	154	20
29	152	146	18	192	157	19
30	155	150¶	19	190	160	19
31	152	154	18	187	157	9
June						
1	146	151**	17	184‖	152**	16
2	147	160	26	187	154	23
3	147	172	24	181	159	15
4	141††	180	25	174††	167	22
5	145	184	22	180	179	30
6	150	189	32	180	187	27
7, 9.30 a.m.	146‡‡	192	28	173‡‡	189	28
5 p.m.	164	217		192	207	
8, 9 a.m.	162	223	49	185	213	42
1.30 p.m.	163	234	18§§	185	221	16§§

TABLE VI—*Concluded.*

Date.	Female 4590.†	Litter.	Food consumed during previous 24 hrs.	Female 4591.†	Litter.	Food consumed during previous 24 hrs.
June, 1927	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
8, 4.15 p.m.	167	240‖‖	12§§	193	233	15§§
9, 9.15 a.m.	164	245	13§§	191	233	22§§
11.50 a.m.	168	252	22§§	192	242‖‖	21§§

* For method of preparation, see text.

† Females 4590 and 4591 had litters of seven and eleven young respectively and were allowed six each to rear.

‡ 500 mg. of Harris yeast to mothers daily.

§ Food consumed during a 48 hour period.

‖ Young opened their eyes.

¶ Young eating.

** 500 mg. of Harris yeast given to litters instead of to mothers, daily.

†† Distributed Harris yeast between mothers and young, allowing 100 mg. to mothers and 400 mg. to litters daily.

‡‡ Replaced Harris yeast by Lilly Yeast Concentrate P-19618, allowing 100 mg. to mothers and 400 mg. to litters daily.

§§ Food consumed during short intervals indicated.

‖‖ Young weaned.

date of birth of the litters, we have been able in a good many cases to prolong lactation for at least a week or 10 days, the young subsequently reaching a maintenance curve or developing marked symptoms of beriberi, characterized by posterior paralysis, muscle chills, and screaming, running fits. On June 1, when the litters of Females 4590 and 4591 (Table VI) reached a maintenance curve followed by slow loss of body weight, the 500 mg. daily dosage was removed from the mothers and given to the litters. The responses of the nursing young were quite definite, accompanied by increases in food consumption. Since the mothers had lost weight by such procedure, on June 4 the 500 mg. daily dosage was distributed between mothers and young, allowing 100 mg. to the mothers and 400 mg. to the litters. Such change resulted in slow growth of mothers and young. On June 7, when it became apparent that the litter of Female 4590 gained only 3 gm. and the litter of Female 4591 gained only 2 gm. during the previous 24 hours, the Harris yeast was replaced by the Lilly Yeast Concentrate P-19618, allowing the same daily dosage and same distribu-

tion between mother and young. Having already had available data on the potency of the Lilly yeast concentrate, we expected marked responses of the nursing young; consequently detailed records were made at frequent intervals. The striking results secured, however, were even beyond our expectations. In 7½ hours the litter of Female 4590 gained 25 gm. and the mother 18 gm. a total gain for mother and young of 43 gm., and at 4.15 p.m. on the day following the administration of the potent yeast concentrate the litter was weaned. In less than 31 hours the litter gained 48 gm. and the mother 21 gm., a total gain for mother and litter of 69 gm. On our Stock Diet 1, on which we rear over 95 per cent of all our animals for experimental work, we have determined by accurate daily records that it would have taken 72 to 96 hours to wean this litter from the day it collectively weighed 192 gm., when vitamin therapy of the potent concentrated product was initiated. In other words, we have expedited rearing of young on a synthetic diet composed of purified food substances with the aid of a potent vitamin B concentrate, so that growth in the nursing young was accelerated at a rate 2 to 3 times that observed during the last 7 years in stock animals on what we had hitherto considered optimum diets for all physiological functions.

In the case of the litter of Female 4591 the results were similar, but not quite so striking. In 7½ hours the litter gained 18 gm. and the mother 19 gm., a total gain for mother and young of 37 gm. In 30 hours and 45 minutes mother and litter gained a total of 64 gm. It took 50 hours, however, to wean this litter.

By careful analysis of the food consumption records taken at frequent intervals, it will be noted that the unusual rapid gains in body weight are entirely out of proportion to the increased intake of food.

DISCUSSION.

Detailed evidence has been submitted in this communication showing that infant mortality can be entirely circumvented on synthetic diets composed of purified food substances (adequate in every respect except in vitamin B) by a distribution of vitamin B between mother and young according to needs. By use of such technique, not only is mortality of the nursing young overcome, but rearing of the young is at the same time expedited, provided

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the litters receive such amounts of vitamin B as meet their optimum requirements. We have reported findings not only with dehydrated yeast, but also with yeast concentrates, and from the work so far completed it is evident that the lactating mother wastes at least 60 per cent of vitamin B in the metabolism of transfer to the milk. By drawing out a medicine dropper to a fine capillary, we have succeeded in saving nursing young, 10 to 12 gm. in weight, still blind, and constantly nursing. The young, therefore, must have been deriving all the necessary dietary essentials, other than vitamin B, from their mother's milk.

Since we have found at autopsy that the nursing young at the point of death have their stomachs well distended with curd, it is quite possible that the convulsions are due to a gastritis produced by the undigested curd irritating the gastric mucosa. Necropsy findings of a good many of the dying young showed hemorrhages in the intestinal tract which was filled with gas, and hemorrhages in the bones (6). Recently Moore, Brodie, and Hope (7) have reported similar findings, in nursing young, on diets adequate in vitamin B for growth but deficient for lactation.

Our observations on the accelerated growth of nursing young following administration of potent concentrated preparations of vitamin B are in harmony with the recent findings of Osborne and Mendel on accelerated growth in the growing animal on diets abundant in vitamin B (8).

A considerable literature has accumulated on the dual nature of vitamin B, references to which are given in a recent article of Sherman and Axtmayer (9). All the evidence reported points to the fact that the dietary complex designated as vitamin B is composed of two separate factors,—the antineuritic and the growth-promoting. It is quite clear from the data submitted herein that the concentrated vitamin B preparations employed in our investigation are abundant in both the antineuritic and growth-promoting substances.

For years the author has found that a milk powder diet, containing as much as 50 per cent dried skimmed milk, must be fortified with yeast in order to furnish enough vitamin B for normal lactation (10).

Steenbock, Kent, and Gross (11) in their studies on "The Dietary Qualities of Barley" have pointed out that, while barley

fed at a 20 per cent plane of intake, as a source of vitamin B, produces satisfactory growth, even as high a plane of intake as 40 per cent is inadequate for the rearing of young. They apparently encountered similar convulsive conditions in their nursing young, for they state: "They appeared to be paralyzed posteriorly, yet spasmodically without any apparent cause they would dart up the sides of the cage in the most excited manner. For 2 days they were given cow's milk with a medicine dropper in addition to what nourishment they got from their mothers, but with no resultant improvement."

Recently Macy, Outhouse, Graham, and Long (12) have concluded from quantitative investigations that human milk (pooled) obtained from wet-nurses subsisting on the average American diet, although abundant in fat-soluble vitamins, is even more deficient than cow's milk in vitamin B, and suggest that the human nursing mother is probably on the border line with respect to vitamin B. Guest, Nelson, Parks, and Fulmer (13) came to similar conclusions from their studies of the cereals as the only source of vitamin B for fertility and lactation.

When we consider that the American diet is composed largely of degerminated cereals, sugar, and meat (14), it is very questionable whether the human mother is able to secrete sufficient amounts of vitamin B to meet the optimum requirements of nursing infants. From the work reported in this paper it is suggested that a great proportion of the infant mortality during the 1st year of life, associated with gastrointestinal disturbances, may be due to vitamin B deficiencies. Such deficiencies may be brought about by the character of the present American diet and by the physiological inability of the nursing mother to secrete vitamin B quantitatively in the milk. While beriberi in an accentuated form is seldom encountered in this country in the human baby, a good many secondary pathological disturbances and a susceptibility to infection, due to vitamin B deficiencies, may be quite common; at least, it is a problem that needs to be investigated extensively by pediatricians.

SUMMARY.

1. By removing the daily yeast dosage (as a source of vitamin B) from the lactating mother during the latter part of lactation, when the nursing young are failing or when they show prolonged

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maintenance, rearing of the young was expedited and infant mortality at the same time circumvented.

2. Upon applying the same technique with yeast concentrates, it was discovered that the lactating mother dissipates at least 60 per cent, if not more, of the vitamin B dosage daily apportioned to it in the metabolism of transfer to the milk.

3. To avoid infant mortality, nursing young need large amounts of even highly concentrated products of vitamin B daily during lactation.

4. The large requirements of vitamin B for lactation can now be explained as due to the inability of the nursing mother to secrete vitamin B quantitatively in the milk.

5. Accelerated growth of nursing young can be obtained by administration of liberal quantities of potent concentrated preparations of vitamin B.

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A NOTE ON THE CRYSTALLIZATION OF FREE ARGININE AND HISTIDINE.*

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Although both arginine and histidine were obtained in the crystalline condition (1, 2) many years ago, no illustrations of the crystals appear to have been published. In the course of our work upon the separation of arginine and histidine from proteins we have had occasion to prepare highly purified specimens of these bases and, since they are only infrequently seen in the crystalline form, photomicrographs are here submitted.

Both arginine and histidine crystallize readily even from somewhat impure solutions but pure material should be employed if typical crystals are desired. Arginine crystallizes from water as a dihydrate in rectangular prisms which frequently show pyramidal faces (Fig. 1). The mother liquor is not sirupy and at 21° contains approximately 15 per cent of anhydrous arginine. The solubility of this base is therefore much less than that of lysine (3) which crystallizes only from a thick aqueous sirup.

From 66 per cent alcohol arginine separates in thin anhydrous plates with parallel sides (Fig. 2) and irregular ends. These often aggregate into rosettes.

Histidine usually crystallizes from water in thin, lustrous, parallel-sided plates frequently forming rosettes. The plates are so brittle that satisfactory specimens for photography are difficult to secure. Thicker and better developed crystals were obtained by slow crystallization from a somewhat dilute solution (Fig. 3).

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

The crystals are anhydrous, deeply striated, and, except where broken, have very irregular ends.

The crystals of histidine which separate from a saturated aqueous solution on the addition of an equal volume of alcohol are usually small but well developed rectangular plates, the corners of which are frequently cut off so as to form an octagonal outline (Fig. 4). These also aggregate into rosettes and are anhydrous.

Crystallization of Arginine.—Arginine was prepared from edestin (4), purified as the picrate (174 gm.) (5), and a solution containing the free base was obtained with as little exposure of the alkaline fluid to the air as possible. The perfectly clear and colorless solution was divided into two roughly equal parts and concentrated *in vacuo* until crystals began to form on the walls in the upper part of the flask. On cooling arginine separated in rectangular prisms, with occasional crystals 4 to 5 mm. long (see Fig. 1). When crystallization has become complete an increase in temperature of a few degrees is sufficient to etch the crystals and spoil the sharp outlines. Indications of such etch marks can be seen in the figure.

The clear transparent prisms were rapidly filtered, washed with a little ice-cold water, and dried by centrifugation in a Richards crucible. No appreciable amount of carbonate was present. The crystals contain 2 molecules of water of crystallization; theory for $C_6H_{14}O_2N_4 \cdot 2\ H_2O$, 17.14 per cent, found 16.90 per cent. When the prismatic crystals are washed with absolute alcohol and ether and then dried *in vacuo* over lime they become white and opaque with the loss of approximately 1 molecule of water of crystallization. The second molecule is readily driven off at 105°; theory for $C_6H_{14}O_2N_4 \cdot H_2O$, 9.37 per cent, found 9.79 per cent. The anhydrous arginine contained 32.17 per cent of nitrogen, theory 32.18 per cent. Free arginine decomposes at 238° (Anschütz thermometer) on slow heating after darkening from 233°. With more rapid heating decomposition points as high as 244° were obtained but the lower temperature mentioned was characteristic and readily reproducible.

The second portion of the arginine solution was concentrated like the first, warmed until all crystalline material had redissolved and 2 volumes of absolute alcohol purified by refluxing over potassium hydroxide were added. Since no separation occurred

on standing overnight, a small amount of ether was added. A rapid crystallization then took place, the arginine separating in thin

FIG. 1. Arginine (dihydrate), from water. × 60.

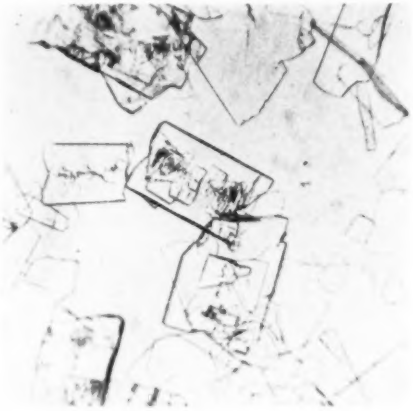

FIG. 2. Arginine (anhydrous), from 66 per cent alcohol. × 75.

transparent parallel-sided plates (Fig. 2) which were often aggregated into radiating masses. The plates were anhydrous and

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contained no carbonate. They were filtered and washed with absolute alcohol and ether; nitrogen 32.11 per cent, theory 32.18 per cent; when heated they behaved exactly like the hydrated

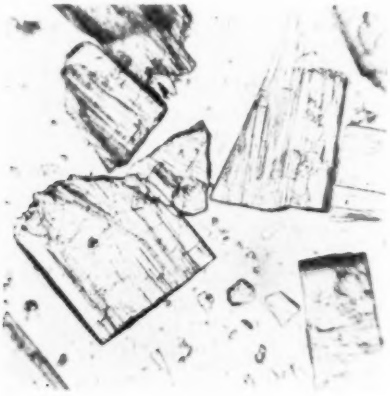

FIG. 3. Histidine, from water. × 60.

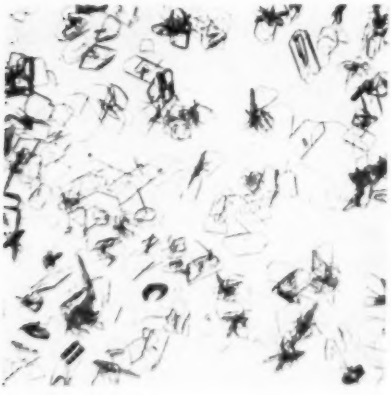

FIG. 4. Histidine, from 50 per cent alcohol. × 130.

form. This method of preparation is to be recommended for its convenience. Although the crystals appear to be reasonably stable in air they should be sealed in glass for preservation.

Crystallization of Histidine.—Histidine was prepared from blood cells by the method of Hanke and Koessler (6) and was crystallized twice as chloride, once as dinitronaphtholsulfonate and twice as free base (5) from 50 per cent alcohol. It was again crystallized from about 10 parts of water when it separated in fairly thick, striated, brittle plates decomposing at 277° on slow heating (Fig. 3).

A second preparation which had been crystallized twice as chloride, and twice as free base, when recrystallized from about 7 parts of water gave thin, extremely brittle, anhydrous plates decomposing at 277–278° on slow heating and containing 27.09 per cent of nitrogen; theory for $C_6H_9O_2N_3$, 27.10 per cent. The addition of an equal volume of absolute alcohol to the aqueous liquor gave rise to the crystallization shown in Fig. 4 in which typical rosette aggregates can be seen.

SUMMARY.

Arginine crystallizes from water in prisms of the dihydrate (Fig. 1) and from 66 per cent alcohol in anhydrous plates (Fig. 2). Histidine crystallizes from water (Fig. 3) and from 50 per cent alcohol (Fig. 4) in thin anhydrous plates.

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MODIFICATIONS OF THE METHOD FOR THE DETERMINATION OF THE BASIC AMINO ACIDS OF PROTEINS. THE BASES OF EDESTIN.*

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(Received for publication, January 17, 1928.)

The accurate determination of the basic amino acid constituents of proteins is a problem that is assuming considerable importance. Thoroughly exact figures would have much significance in the calculation of the minimal molecular weights of proteins as well as in many physiological investigations. Moreover a development of existing methods is essential before they can be conveniently applied to such problems as the investigation of the "basic nitrogen" of extracts from biological material. We have therefore studied the conditions under which a quantitative estimation of the bases in proteins may be made and have attempted to shorten the procedure of Kossel and Kutscher (1) by conducting the silver precipitations under somewhat different and more precise conditions and by avoiding the use of nitrates; further, the determinations of arginine and of histidine have been based upon the weights of the crystalline dinitronaphtholsulfonates instead of upon nitrogen determinations in the respective fractions. The determination of lysine is, as has always been customary, based on the weight of its picrate. The method that has been employed may be indicated as follows:

1. The protein (at least 50 gm.) is hydrolyzed by boiling for 30 hours with 20 per cent hydrochloric acid; the hydrolysate is repeatedly concentrated to a sirup *in vacuo* to remove as much acid as possible.

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

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2. The sirup is diluted to about 2000 cc. and carefully treated with silver oxide in suspension until the chloride is completely precipitated, the solution being maintained *distinctly acid to Congo paper* by the addition of dilute sulfuric acid as required. The silver chloride is centrifuged off and thoroughly washed with hot water.

3. The solutions and washings are concentrated to approximately 2000 cc. and silver oxide and sulfuric acid added as before until a sample of the fluid gives a buff-colored precipitate of silver oxide when tested on a watch-glass with barium hydroxide. Warm saturated barium hydroxide solution is then added until the solution is strongly alkaline to phenolphthalein paper. The precipitate is centrifuged off and washed once with water made alkaline with barium hydroxide. The filtrate and washings are acidified to Congo paper with sulfuric acid, silver is removed as sulfide, and the fluid is concentrated. This solution is subsequently worked up for the *lysine fraction.*

4. The precipitate, which contains histidine and arginine as silver compounds, is suspended in water, acidified with sulfuric acid, and the silver compounds decomposed with hydrogen sulfide on a shaking machine. The silver sulfide is removed and washed and the solution concentrated to approximately 2000 cc.

5. Silver oxide is added in excess to this solution, the acidity being maintained as before. Cold saturated barium hydroxide is then slowly added with constant stirring until the solution has been brought to pH 7.0 as shown by brom-thymol blue, whereby the histidine is completely precipitated.[1] The precipitate is centrifuged off, suspended in water, sufficient hydrochloric acid to effect complete decomposition is added, and the solution boiled. Silver chloride is removed and thoroughly washed and to the solution and washings silver oxide and sulfuric acid are added in excess, as before. The solution is again brought to pH 7.0, centrifuged, and the precipitate decomposed with dilute hydrochloric acid. This gives the crude *histidine fraction.*

6. This solution is concentrated to dryness *in vacuo* several times to remove excess of hydrochloric acid, diluted, and the remaining hydrochloric acid removed with silver oxide and sulfuric acid,

[1] Some of the technical details of these operations are more fully described in previous papers (2, 3).

the excess of silver precipitated with hydrogen sulfide, the solution concentrated to about 300 cc., and sufficient sulfuric acid to make 5 per cent added. The histidine is then precipitated by the addition of an excess of Hopkins' mercuric sulfate reagent. After standing for 48 hours, the precipitate is centrifuged, washed with diluted reagent, and decomposed with hydrogen sulfide. The filtrate is discarded. After removal of the mercuric sulfide and slight concentration, barium hydroxide is added until only a trace of sulfuric acid remains. The barium sulfate is removed and the solution made up to 500 cc. This is the purified *histidine fraction*. An aliquot is taken for a nitrogen determination from which the necessary amount of dinitronaphtholsulfonic acid may be calculated (1 gm. of histidine nitrogen requires 14.96 gm. of reagent, but a slight excess should be used). A large aliquot, or the remainder of the solution, is concentrated to approximately 50 cc., the reagent added to the hot solution, and the di-salt of histidine and dinitronaphtholsulfonic acid (4) crystallized in two successive crops. The weight of the anhydrous di-salt multiplied by 0.1979 gives the equivalent of histidine. This must be corrected for the aliquot removed for analysis.

7. The filtrates from the two silver precipitations made at pH 7.0 are combined, acidified to Congo paper with sulfuric acid, and concentrated *in vacuo* to approximately 2000 cc. Warm saturated barium hydroxide is added until the solution is strongly alkaline to phenolphthalein paper and the precipitate of arginine silver is centrifuged off and washed with alkaline water. Silver and barium are removed from the filtrate which is then concentrated and added to the *lysine fraction* (see Section 3).

The precipitate is suspended in water, acidified to the *faintest possible* reaction of Congo paper with sulfuric acid and decomposed with hydrogen sulfide. The filtrate from the silver sulfide, together with the washings, is concentrated to 500 cc. and nitrogen determined in an aliquot. This is the *arginine fraction*. A suitable aliquot of this solution is treated at boiling temperature with a small excess over the requisite amount of dinitronaphtholsulfonic acid (1 gm. of arginine nitrogen requires 5.61 gm. of reagent) and the arginine compound crystallized in two successive crops. The weight of this compound multiplied by 0.3566 gives the equivalent of arginine which must be corrected for the aliquot taken for nitrogen determination.

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8. The combined crude lysine fractions contain the ammonia arising from the hydrolysis of the protein. To remove this the solution is made alkaline with barium hydroxide, the barium sulfate is centrifuged off and washed, and the solution concentrated *in vacuo* until all the ammonia has passed off. The addition of alcohol facilitates this operation. The residue is acidified with sulfuric acid, barium sulfate is centrifuged off, and the solution brought to approximately 500 cc. Sufficient concentrated sulfuric acid to make 5 per cent is added and the lysine is precipitated by the addition of an excess of 20 per cent phosphotungstic acid in 5 per cent sulfuric acid. After standing overnight the precipitate is centrifuged, washed twice with 2 per cent phosphotungstic acid in 5 per cent sulfuric acid, and dissolved in a liberal quantity of acetone. The acetone solution is diluted with water and the phosphotungstic acid precipitated by the slow addition of barium hydroxide. The barium phosphotungstate is washed once in the cold and subsequently boiled up twice with dilute barium hydroxide. The combined solution and washings must be strongly alkaline to phenolphthalein paper and clear. Barium hydroxide is exactly removed by the requisite quantity of sulfuric acid, the clear solution brought to 500 cc., and nitrogen determined in an aliquot. This is the *lysine fraction.*

9. It is concentrated to a thin sirup, treated with sufficient absolute alcohol to produce a faint turbidity, and saturated alcoholic picric acid added until the precipitation of lysine picrate is complete; excess must be carefully avoided. After standing overnight the lysine picrate in filtered off, washed with absolute alcohol and with ether, dried, and weighed. If impure it should be recrystallized from a minimal amount of hot water, the filtrate measured, and a solubility correction of 0.54 gm. per 100 cc. of aqueous mother liquor added to the weight of the recrystallized picrate. The weight multiplied by 0.3895 gives the yield of free lysine. Satisfactory picrate should decompose with a slight explosion above 250°.

Discussion of Procedure.

Hydrolysis of the protein by boiling 20 per cent hydrochloric acid is by far the most convenient procedure in view of the subsequent operations. The 30 hour period recommended should

be sufficient but should be controlled in doubtful cases by a determination of the ratio of amino to total nitrogen. Hydrolysis must be continued until this reaches a maximum.

The use of a suspension of silver oxide together with sulfuric acid in place of a solution of the nitrate possesses many advantages. It is essential to add small quantities at a time with constant, preferably mechanical, stirring. Serious losses of histidine may occur if the solution is allowed to become alkaline during the removal of hydrochloric acid; histidine silver, once precipitated, does not readily redissolve in dilute sulfuric acid. It is best to add a liberal amount of sulfuric acid at the beginning. If doubt arises on this point the silver chloride should be extracted with hot water to which a little hydrochloric acid has been added and the operations of removal of acid and precipitation of the histidine repeated on this extract. The arginine and histidine are precipitated together as a first step in order to reduce as far as possible the gross impurity of the histidine fraction.

The double precipitation of histidine at pH 7.0 has been shown to be necessary (2) in order to effect complete separation of the two bases. A little trouble is usually experienced in bringing the reaction to pH 7.0 the second time as this solution is only slightly buffered. Some latitude is, however, permissible at this point.

The purification of the histidine fraction by Hopkins' reagent is necessary. The best alternative is the use of phosphotungstic acid but, owing to the solubility of histidine phosphotungstate, this involves the recovery of histidine from the filtrate by means of silver and is, moreover, a much more laborious process. The direct crystallization of the dinitronaphtholsulfonate from the crude histidine fraction is not satisfactory.

The two acid filtrates containing the arginine together with an excess of silver sulfate may be concentrated *in vacuo* without trouble and decanted from any silver sulfate that separates. If this does not occur a test for the presence of excess silver should be applied before proceeding with the precipitation of arginine silver. The arginine fraction is usually about 90 per cent pure. The separation of the second small crop of the dinitronaphtholsulfonate is impossible if too much sulfuric acid is added to the arginine silver before this is decomposed by hydrogen sulfide.

Great care must therefore be exercised at this point. This second crop usually crystallizes in yellow needles insoluble in alcohol which are probably a di-salt of arginine and the reagent. On boiling with water the substance is converted into the orange mono-salt which, after standing some time, may be filtered off and weighed. It may, if necessary, be recrystallized from a little dilute acid and obtained in typical plates, but the weight is usually too small to justify this.

TABLE I.

Bases of Edestin.

The results are expressed as proportions of the free base in the protein calculated from the nitrogen or from the weight of the salt, on the assumptions that the nitrogen represented nothing but the individual base and that the salt was pure. The relative purity of the fractions can thus be readily noted.

	Histidine.		Arginine.		Lysine.	
	Total N of fraction.	Weight of salt.	Total N of fraction.	Weight of salt.	Total N of fraction.	Weight of salt.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
	2.20	1.77	15.96	13.95		
	2.19	1.74	15.96	13.72	3.00	1.83
	2.09	1.83	15.11	13.46	3.79	2.12
Average.....	2.16	1.78	15.68	13.71		1.97
Kossel and Patten.	2.36		13.97			1.67
	2.10		14.36			1.63
	2.10					
Average.....	2.19		14.17			1.65

The lysine fraction obtained by precipitation with phosphotungstic acid in the presence of all the other amino acids is grossly impure, one-half of its nitrogen frequently belonging to amino acids other than lysine. The recrystallization of the picrate from this solution is not always necessary, however, as we have often obtained lysine picrate precipitates that decomposed at temperatures above 260° and were, consequently, perfectly satisfactory to weigh. The phosphotungstic acid procedure for the isolation of lysine is undeniably cumbersome. The technique described is the least troublesome we have used, but a new reagent that will

selectively precipitate lysine from this mixture is greatly to be desired.

TABLE II.

Bases of Edestin.

The results are expressed as proportions of free base in the protein calculated from the weight of nitrogen or of salt.

	per cent	*per cent*	*per cent*
Histidine.			
N in main fraction	5.49		
" " phosphotungstic acid precipitate	2.17		
Dinitronaphtholsulfonate from phosphotungstic acid precipitate		1.65	
N in Ag precipitate from phosphotungstic acid filtrate	0.64		
Dinitronaphtholsulfonate from Ag precipitate from phosphotungstic acid filtrate		0.43	
Total histidine		2.08	
Histidine by Van Slyke's method			3.98
Arginine.			
N in main fraction	15.82		
Picrate from main fraction		14.81	
Dinitronaphtholsulfonate from mother liquor of picrate		0.26	
N in second Ag precipitate	0.60		
Dinitronaphtholsulfonate from second Ag precipitate		0.30	
Dinitronaphtholsulfonate from lysine fraction		0.39	
" " final lysine picrate mother liquors		0.05	
Total arginine		15.81	
Arginine by Van Slyke's method			15.8
Lysine.			
N in main fraction	4.85		
Picrate from main fraction		1.94	
N in phosphotungstic acid precipitate in filtrate from arginine silver	0.40		
Picrate from this phosphotungstic acid precipitate		0.25	
Total lysine		2.19	
Lysine by Van Slyke's method			3.75

Analysis of Edestin.

Edestin was selected for the present investigation as no analysis of the bases in this protein by the direct method has been attempted since the early work of Kossel and Patten (5). The protein is so readily obtained and so frequently used in various investigations that it seemed advisable to subject it to a more thorough examination. The results of the analysis are given in Table I. According to the procedure formerly employed the proportions of arginine and histidine were calculated from the nitrogen content of their respective fractions on the assumption that these bases had been quantitatively separated in pure form. A comparison of the proportions as calculated from the weights of the crystalline derivatives with those calculated from the nitrogen content shows that this assumption is not entirely correct.

Large Scale Analysis of Edestin.

Although the above figures for the proportions of the three bases in edestin are in fair agreement with the earlier work of Kossel and Patten, the much higher results obtained by Van Slyke (6) (Table II) appear to indicate that they may be somewhat low. Further, during the development of the method for preparing arginine (3) 14.45 per cent of edestin was isolated as crystalline compounds of arginine. This indicated that the use of larger amounts of the protein favored a more complete separation of the basic constituents. We have therefore repeated the analysis using 366.3 gm. of the protein, the larger quantity permitting a more detailed examination of the various fractions. The results are given in Table II.

The histidine fraction was, in this case, treated with phosphotungstic acid and the precipitate worked up in the usual way, the histidine being weighed as dinitronaphtholsulfonate. The filtrate from the phosphotungstic acid precipitate was freed from reagents and treated with silver and barium hydroxide at a strongly alkaline reaction in the hope that the silver compounds of the amino acid impurities which precipitate at pH 7.0 might be soluble at a more alkaline reaction. A large part of them was indeed thus eliminated but the small histidine fraction secured was still far from pure. This method is not to be recommended.

Purification by Hopkins' reagent is much simpler and more satisfactory.

The arginine silver was precipitated from a volume of 11.3 liters and the filtrate freed from reagents and concentrated to 4 liters. To insure the removal of as much of the arginine as possible this was again treated with silver and barium hydroxide, and the small precipitate worked up separately. Notwithstanding the care taken with these precipitations as well as with the preliminary precipitation of the histidine and arginine silver, a little arginine was subsequently found in the lysine fraction from which it was isolated by means of dinitronaphtholsulfonic acid in the usual way. Further experimentation will be necessary before it can be certain whether this small quantity of arginine represented a solubility of arginine silver in the mixture of amino acids from which the precipitations were made or whether, through some inadvertency, insufficient silver was used. It is obvious that the complete precipitation of arginine presents serious difficulties.

When the results of this analysis are compared with those on smaller amounts of protein given in Table I it appears that the histidine and arginine content of the protein here found agree very closely with that indicated by the nitrogen content of the respective fractions in the former analyses, a circumstance which might lead to the tempting conclusion that such nitrogen determinations give results actually closer to the true content of the bases in the protein than do the weights of the derivatives. This might be justified if it were not certain that such fractions contain amino acid impurities. It would be highly dangerous to trust implicitly in such a fortunate compensation of errors. It is probable that the unavoidable losses which occur in this type of work told more heavily when the smaller quantities of protein were used. The data on the recovery of histidine and arginine given in an earlier paper (2) indicate that one may expect to recover approximately 90 per cent of these two bases as crystalline derivatives when working with relatively small quantities. Making this assumption the small scale analyses indicate the presence of approximately 2.0 per cent of histidine and 15.2 per cent of arginine in edestin, results which agree reasonably well with the large scale analysis. A similar correction applied arbi-

trarily to the lysine determination gives 2.2 per cent, bringing this also into close agreement with the results of the large scale analysis. Unfortunately we have no alternative method for the isolation of this base and there is, consequently, little opportunity to judge the true worth of lysine determinations.

It will be noted that the arginine determination in this analysis checks that of Van Slyke closely and our results therefore support Plimmer's contention (7) that arginine determinations by the indirect method are accurate. The results for histidine are, however, much lower than Van Slyke's and do not even approach Hanke and Koessler's figure of 3.04 per cent obtained by a colorimetric method (8). A final decision regarding the true histidine content of edestin cannot yet be made but it seems improbable that nearly half of the histidine present in our solutions could have escaped isolation. Our lysine figure is also lower than Van Slyke's and here again final judgment must be suspended until a better method for the quantitative isolation of lysine has been developed.

It is obvious that the determination of the bases in proteins is still far from being the precise operation that might be desired. The wide discrepancies between the results of different methods of analysis indicate that further study is necessary.

Use of Phosphotungstic Acid as a Preliminary Precipitant.

In many of the published analyses of the basic amino acids of proteins precipitation by phosphotungstic acid has been employed as a preliminary step upon the assumption that no material proportion of the bases passes into the filtrate. That this assumption is not justified with respect to arginine and histidine is shown by the results of an experiment in which the filtrate from such a precipitation was examined for these bases. Owing to the inevitable losses in the greater number of operations, the total yields of the salts of histidine and arginine are somewhat low. The data are given in Table III, and indicate that very considerable losses of histidine and arginine may occur if no steps are taken to recover them from the filtrate from the phosphotungstates. Application of the corrections for the solubility of the phosphotungstates employed by Van Slyke (6) does not materially help the situation. In this experiment 49.55 gm. of edestin were used and the filtrate

from the phosphotungstic acid precipitate measured 3300 cc. This filtrate should therefore contain 3300 × 0.000019 gm. of histidine nitrogen or 0.231 gm. of histidine, and 3300 × 0.000016 gm. of arginine nitrogen or 0.166 gm. of arginine. These quantities correspond to 0.46 per cent of histidine and 0.33 per cent of arginine in the protein. Thus more than twice as much arginine as was to be expected was isolated from the filtrate, while less than half of the expected histidine was found. This may mean that we were unable to obtain in crystalline form all of the histidine actually present in the phosphotungstic acid filtrate; but it also indicates that the solubility of arginine phosphotungstate under these conditions has been underestimated.

TABLE III.

Data showing the presence of arginine and histidine in the filtrate from the phosphotungstic acid precipitate of the bases of edestin. The results are expressed as proportions of free base in the protein.

	Histidine.		Arginine.	
	Total N of fraction.	Weight of salt.	Total N of fraction.	Weight of salt.
	per cent	*per cent*	*per cent*	*per cent*
Phosphotungstic acid precipitate	1.69	1.30	13.51	12.44
Filtrate	0.51	0.20	1.16	0.75
Total	2.20	1.50	14.67	13.19

Plimmer and Lowndes (9) have recently suggested the possibility that a part of the arginine is racemized during the hydrolysis of the protein and that racemic arginine phosphotungstate may be more soluble than the salt of the active form. That this is improbable was shown by an examination of the arginine dinitronaphtholsulfonate obtained from this filtrate. The salt was decomposed with hydrochloric acid, the dinitronaphtholsulfonic acid was extracted by butyl alcohol, and hydrochloric acid added to the colorless solution to a concentration of 20 per cent. The specific rotation of the arginine in this solution was $[\alpha]_D^{20} = +25.6°$, in excellent agreement with the findings of Gulewitsch (10).

EXPERIMENTAL.

Since practically all of the operations in the above procedure are familiar to those having experience with the methods of protein analysis it is unnecessary to describe them in greater detail. Certain points may, however, be discussed more fully.

Isolation of Arginine Dinitronaphtholsulfonate.—The recovery of this compound from a solution which contains other nitrogenous substances presents some difficulty as only a small excess of the reagent over the amount required for the formation of the mono-salt with the arginine should be added. The second precipitate of arginine silver obtained in the large scale analysis furnished a solution which contained half of its nitrogen in forms other than arginine. The solution (500 cc., 0.71 gm. of nitrogen) was treated at boiling temperature with a 10 per cent solution of dinitronaphtholsulfonic acid, small amounts being added at a time. Crystallization occurred promptly, especially along the lines of scratches made by the stirring rod. The reagent was added to the warm solution until, when 2 cc. in addition were added, this crystallization could no longer be detected. After standing overnight the crystals were removed and the mother liquor concentrated. A further addition of 2 cc. of reagent gave, on long standing, a second crystalline separation of only 0.15 gm. while the first crop weighed 2.78 gm. When corrected for the aliquot previously removed for nitrogen this was equivalent to 0.30 per cent of the protein. The crystals corresponded in appearance, solubility, and behavior on heating, with authentic arginine dinitronaphtholsulfonate. Unfortunately it is difficult to identify this substance more precisely as it does not melt satisfactorily and nitrogen determinations by modifications of the Kjeldahl method are uncertain.

Isolation of Arginine Picrate.—The main arginine fraction from the large scale analysis contained 18.7 gm. of nitrogen in 2000 cc. of which 1995 cc. were concentrated to 500 cc. and 70 gm.[2] (76.4 = 1 mol) of dry picric acid were added. Arginine picrate separated promptly on cooling but the solution was allowed to stand 2 days before filtration. The crystals were washed with 70 per cent

[2] These arginine fractions are usually about 90 per cent pure. If the full molar quantity of picric acid is added we have experienced much difficulty in separating pure second crops of crystals from the concentrated mother liquor.

alcohol and then with absolute alcohol. Dried at 110° this crop weighed 110.0 gm., decomposed at 217–218°, and was therefore pure. The mother liquor and washings, on concentration to 100 cc. yielded a second crop of 13.6 gm. and the second mother liquor, 1.9 gm. of somewhat impure material. Since the further addition of picric acid gave impure crystals the final mother liquor was acidified with sulfuric acid, freed from picric acid with ether, and neutralized to Congo paper with barium hydroxide. Barium sulfate was then removed and the concentrated solution treated as above described with a 10 per cent solution of dinitronaphtholsulfonic acid. Two crops of the arginine compound weighing 2.66 and 0.63 gm. respectively were obtained in pure form. The corrected equivalents of these several crops of arginine compounds expressed as proportions of arginine in the protein are given in Table III.

Isolation of Lysine Picrate.—The main lysine fraction from the large scale analysis was treated according to the customary procedure whereby 17.8 gm. of crude lysine picrate were obtained. On recrystallizing once this weighed 15.02 gm., not corrected for solubility, and decomposed at 266°. This is equivalent to 1.61 per cent of the edestin. The mother liquor from the crude picrate was freed from reagents and the base present was precipitated by phosphotungstic acid. The crude picrate from this material was extensively recrystallized; all mother liquors were combined and the base contained in them again precipitated with phosphotungstic acid and converted to picrate. In all 7.09 gm. were secured, the crystalline form of which suggested that of impure arginine picrate. The material was therefore decomposed in the usual way with sulfuric acid and ether and the arginine precipitated with dinitronaphtholsulfonic acid, whereby arginine salt equivalent to 0.39 per cent of edestin was obtained. The sulfonate mother liquors were freed from reagents and treated with picric acid in the usual way. Lysine picrate equivalent to 0.33 per cent of the protein, and decomposing above 250° was separated, making a total yield of lysine of 1.94 per cent of the protein from this fraction.

The mother liquors from which the above mentioned 7.09 gm. of mixed arginine and lysine picrate had been separated were worked over for arginine dinitronaphtholsulfonate. A small quantity equivalent to 0.05 per cent of the protein was isolated.

The isolation of the lysine from the filtrate from the arginine silver presented no difficulties. The recrystallized picrate weighed 2.27 gm. (corrected) and decomposed at 266–267°. When corrected for aliquots removed this was equivalent to 0.25 per cent of the protein.

Histidine Fraction.—The main histidine fraction from the large scale analysis contained 5.44 gm. of nitrogen, of which less than half belonged to histidine. It was reddish brown in color. The histidine was precipitated by phosphotungstic acid in the presence of 5 per cent hydrochloric acid and the precipitate allowed to stand in the cold for several days. The phosphotungstate was then filtered off, washed, dissolved in acetone, and decomposed with barium hydroxide. The barium phosphotungstate was carefully extracted several times with hot dilute barium hydroxide and the clear solution then treated with sufficient sulfuric acid to remove the barium exactly. The pale yellow filtrate from the barium sulfate was acidified with hydrochloric acid. It contained 2.15 gm. of nitrogen and deposited impure crystals of histidine dichloride when concentrated to a sirup. A one-fifth aliquot, when treated with a small excess over the requisite 2 mols of dinitronaphtholsulfonic acid, yielded 6.44 and 0.97 gm. of di-salt of histidine in two successive crops, equivalent to 1.65 per cent of the protein. The identity of this material was established by comparison with authentic histidine di-sulfonate.

The filtrate from the histidine phosphotungstate was extracted with a mixture of butyl alcohol and ether to remove the greater part of the phosphotungstic acid, concentrated to a sirup, diluted, and the remaining phosphotungstic acid precipitated with barium hydroxide in the customary way. After removal of barium from the filtrate the histidine was precipitated with silver and barium hydroxide at a strongly alkaline reaction to phenolphthalein. The silver compound was decomposed with hydrochloric acid, giving a solution which contained nitrogen equivalent to 0.64 per cent of the protein when calculated as histidine. On treatment with dinitronaphtholsulfonic acid two crops of di-salt weighing together 7.17 gm. were secured, which are equivalent, when corrected for the aliquots removed for nitrogen determination, to 0.43 per cent of the protein. It should be noted that a somewhat lower standard of purity is permissible with this substance

than with lysine picrate inasmuch as the weight of the free histidine is less than one-fifth the weight of the salt. We regard material decomposing above 230° as satisfactory for weighing although the pure substance decomposes at 251°.

The filtrate from the histidine silver precipitate was freed from reagents. On concentration, over 10 gm. of nearly pure aspartic acid separated (N = 10.4 per cent, theory 10.53 per cent). From the mother liquor a second crop (4.7 gm.) of crystals was obtained which were dissolved in a little warm concentrated hydrochloric acid. On standing, glutaminic acid hydrochloride (1.3 gm.) separated, which melted at 209–210° and contained 7.65 per cent of nitrogen, theory 7.63 per cent.

It is interesting to note that a large part of the aspartic acid of the edestin, accompanied by some glutaminic acid, went through the procedure along with the histidine and was only separated from it by a step especially designed to this end. This behavior of aspartic acid is very suggestive and, on further investigation, may lead to a better method for its isolation from protein hydrolysates than we at present possess.

SUMMARY.

The procedure for the determination of the basic amino acid constituents of proteins has been modified with the object of facilitating the analysis and at the same time increasing the trustworthiness of the results. The separation of histidine from arginine is accomplished by taking advantage of the solubility of arginine silver at pH 7.0 at which reaction histidine silver is completely precipitated. The determinations of these two bases are based upon the weights of their compounds with dinitronaphtholsulfonic acid; the determination of lysine is based, as formerly, upon the weight of lysine picrate. Throughout the procedure silver nitrate is replaced by silver oxide which is added to a solution maintained acid with sulfuric acid. The troublesome washing necessitated by the presence of the nitrate ion is thereby avoided.

Present experience indicates that when analyses are carried out with 50 gm. samples of protein the proportions of the three bases indicated by the weights of their respective salts are approximately 90 per cent of the proportions which are found when a much larger

sample of the protein is analyzed. It therefore seems justifiable to increase the proportions so found by one-ninth. A large scale analysis of edestin indicates that this protein yields 2.08 per cent of histidine, 15.8 per cent of arginine, and 2.19 per cent of lysine.

Evidence is presented to show that preliminary precipitation of the three bases by phosphotungstic acid is accompanied by losses of arginine and histidine in the filtrate that are not adequately corrected for by the use of solubility factors that have been widely employed for this purpose.

The unexpected high solubility of arginine phosphotungstate is not due to a partial racemization as the arginine recovered from a phosphotungstate filtrate possesses normal optical activity.

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IODINE IN GORGONIAN CORALS.

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1. Content of Iodine in the Axial Skeleton.

In 1896 Drechsel demonstrated that the horny axial skeleton of the Mediterranean coral, *Gorgonia cavolinii*, contains iodine in organic combination in a nitrogenous substance termed gorgonin by him. Hundeshagen (1895) had previously investigated an iodine-containing organic material which he separated from marine sponges; and later Harnack (1898) isolated from the same source a compound which he termed iodospongin. Other investigators also have more recently studied the presence of iodine in organic combination in different organisms (McClendon, 1927). In 1901 Mendel and subsequently his pupil Cook (1905) demonstrated the occurrence of iodine in gorgonian corals from the region of the West Indies and compared their iodine content with Drechsel's result; in 1909 Wheeler and Mendel isolated the iodine complex in sponge and identified it positively with 3,5-diiodotyrosine.

Through the kindness of Professor Lafayette B. Mendel I have had an opportunity to examine specimens of several species of gorgonian corals which were collected through the assistance of Professor W. H. Longley of the Marine Laboratory of the Carnegie Institution at Tortugas, in June, 1925. Their iodine content has been determined. The method employed was essentially a combination of the procedures of Kendall and Richardson (1920) and Pickworth (1925).

0.25 to 0.50 gm. of fine powdered material (horny axial skeletons carefully separated from the crude adherent concretions of

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inorganic salts) is weighed and introduced into a nickel crucible of 150 cc. capacity; 10 cc. of 50 per cent sodium hydroxide are added and mixed by rotation of the crucible; it is almost covered with a lid, and evaporated on a steam bath. The residue is next heated, at first slowly by placing the crucible within a larger one on a layer of sand; then enough to yield a clear fusion product in about an hour. The crucible is next heated over a large free flame protected so that the crucible and lid can be rapidly raised to a dull red heat, which is maintained ½ to 1 minute. Allow the crucible to cool, add about 70 cc. of water, and place on a warm

TABLE I.

Summary of Trial Experiments.

Material.	Iodine.	
	mg.	per cent
Bath sponge, 1 gm	7.677	0.768
" " 1 "	7.694	0.769
" " 0.5 "	3.838	0.768
" " 0.5 "	3.822	0.764
Iodine solution, 10 cc. (1 cc.:0.1 mg. I)	0.976	
" " 10 " 1 " :0.1 " "	0.976	
" " 10 " 1 " :0.1 " "	1.009	
" " 10 " 1 " :0.1 " "	0.993	
Iodine solution, 10 cc., and same sponge, 0.5 gm	4.818	
" " 10 " " " " 0.5 "	4.811	
Iodine solution, 10 cc., and milk, 5 cc	1.003	
" " 10 " " " 5 "	0.993	

bath for about an hour, when gentle stirring will bring the whole into solution; transfer to a 250 cc. Erlenmeyer flask and add 3 drops of 10 per cent sodium sulfite solution. Acidify with 50 per cent sulfuric acid from a burette (test acidity with strip of litmus paper), add 3 cc. in excess, cool, and add 5 cc. of approximately 0.1 N permanganate; after 3 minutes add a suspension of charcoal. When decolorization is complete filter carefully through a plaited filter paper and wash with a little water; add a crystal of potassium iodide and titrate immediately (Pickworth, 1925) with 0.005 N thiosulfate which is to be restandardized frequently

(Kendall, 1920), a few drops of 0.5 per cent starch solution being added only towards the end of the titration. All the reagents used were chemically pure, and (except the KI) were previously ascertained to be absolutely free from iodine.

Preliminary trials were made several times with samples of the common bath sponge and with known iodine solutions and milk, in order to gain experience and determine how accurate the method is. The results are shown in Table I.

TABLE II.

Summary of Analyses.

Material.	Moisture.*	Iodine in dry matter.	Iodine in adherent concretion.
	per cent	*per cent*	*per cent*
Eunecia crassa	6.2	1.90	Trace.
" *roussani*	6.2	1.62	0
" sp.	9.7	0.97	†
Gorgonia citrina	8.0	1.17	0
" *flabellum*	9.7	0.80	0
" *acerosa*	10.2	0.79	0
Plexaura flexuosa	7.0	2.63	0.002
" *homomalla*	6.4	1.42	0 (?)
" sp.	9.7	1.55	0
Plexaurella dichotoma	3.4	0.11	0
Pseudoplexaura crassa	8.7	0.94	0
" sp.	11.0	1.38	0
Xiphigorgia anceps	8.9	1.58	0
Unidentified coral from 60 fathoms.	6.5	0.27	†
Japanese "Nori."	5.5	0.01	†
" "Kombu."	12.8	0.03	†

* Loss of weight at 110°.

† Not ascertained.

The skeletal substance of the gorgonians identified by Dr. Longley as indicated were then examined with the results shown in Table II.

In Table II the analyses of Japanese "Nori" and "Kombu" are added for comparison. These are not corals but seaweeds, commonly consumed as food in Japan. Analyses of Chinese algæ have recently been published by Read and How (1927).

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In each of the species of gorgonians mentioned above, iodine was found to be a constituent of the horny axial skeletons, but not of the adherent concretions. The content of this element in most of these corals approximates 1.0 to 1.5 per cent.

The preceding observations support the assumption of Drechsel that the gorgonians have a specific iodine metabolism which is essential to the building up of the framework of the axial skeleton, and also give further justification for the belief already stated by Mendel that for many organisms iodine may be as essential an element as is chlorine for others.

2. 3,5-Diiodotyrosine from the Gorgonian Coral, Plexaura flexuosa.

As early as 1848 Vogel suggested that the iodine found in sponges examined by him was present in an organic (not inorganic) form. In 1896 Baumann reported iodine as a normal constituent of the thyroid gland, and in the same year Drechsel isolated "Jodgorgonsäure" from the horny axial skeleton of a gorgonian coral. These classic investigations awakened a new interest in the study of the organic iodine compounds in organisms. Henze (1903) made an examination of the substance described by Drechsel and claimed that it is not iodoaminobutyric acid as had been assumed by its discoverer. Hundeshagen (1895) investigated an iodine-containing organic substance in the marine sponge, and Harnack (1898) demonstrated what he termed "Jodspongin" in the same material to be albuminoid in character. Comparable studies with Harnack's preparation were attempted later by Baumann (1896) and by Rosenfeld (1900). Mendel (1901) and Cook (1905) studied the chemical composition of some gorgonian corals and described the presence of iodine; Scott (1906) examined the nature of the iodine complex in sponges.

However, the identification of the so called iodogorgoic acid was completed for the first time by Wheeler and Jamieson (1905), who established the fact that it is identical with 3,5-diiodotyrosine synthetically prepared by them. Subsequently, Wheeler and Mendel (1909) isolated an iodine compound from the common bath sponge and demonstrated it to be 3,5-diiodotyrosine. It will be recalled that the thyroxin from the thyroid gland is an iodized derivative of tyrosine. What variation may exist in the iodine-bearing complexes in living organisms remains to be ascertained.

I have attempted the identification of the iodine complex in *Plexaura flexuosa*, which was selected for study because of its comparative richness in iodine content.

The material was collected by Dr. Longley of the Carnegie Institution of Washington at Tortugas. The horny axial skeletons which were carefully separated from the crude adherent concretions and finely powdered were used, and the preparation was carried out by the method by which Wheeler and Mendel (1909) isolated 3,5-diiodotyrosine from the common bath sponges.

From 60 gm. of the skeletal material approximately 0.5 gm. of sandy crystals was obtained. It was recrystallized from water. The crystals were seen under the microscope to consist of thin yellowish white plates. The determination of iodine was made by the method previously described.

I. 0.0735 gm. substance gave 0.0429 gm. iodine (58.38 per cent).
II. 0.0608 " " " 0.0355 " " (58.42 " ").

	Calculated for diiodotyrosine, $C_9H_9O_3NI_2$. per cent	Found. per cent
I.....	58.66	58.40

M. p. of diiodotyrosine, 213.0°. M. p. of crystals, 212.7°.

The crystals when warmed with Millon's reagent do not give a red color. A portion was dissolved in strong hydriodic acid and evaporated to dryness on the steam bath. After this reduction the water solution gave a strong test for tyrosine with the above reagent.

The appearance, properties, solubility, *etc.*, of the crystals obtained justify the conclusion positively in every respect that the iodine complex of *Plexaura flexuosa* is identical with 3,5-diiodotyrosine, or the so called iodogorgoic acid.

I am grateful to Professor Lafayette B. Mendel for guidance in this study.

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THE RELATION OF DIET TO THE QUALITY OF FAT PRODUCED IN THE ANIMAL BODY.

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The problem of fat formation in the animal body has long been the subject both of speculation and experimental investigation. If the fats were stored and distributed under the conditions of uniformity with which carbohydrates appear in the body as glucose or glycogen or with which the specific proteins of the tissues and blood seem to occur, the problem would be different from that ordinarily presented in reality. The fats occurring in the tissues and fluids actually vary in chemical composition as well as in the quantities in which they may be present. Not only is the fat of one species usually notably unlike that of another, but even within the species variations in composition may occur from time to time. For example, different samples of lard or of beef suet are not always identical in their chemical make-up. It becomes improbable, therefore, that the fats of the organism are merely synthesized *de novo*. There is much accumulated evidence that they are derivable, in the body, from all of the proximate principles: fats, carbohydrates, and even the nitrogenous proteins (Atkinson, Rapport, and Lusk, 1922). To what extent the fats produced through synthetic procedures, from carbohydrate or protein, are uniform in character; whether food fats are deposited as such, or whether some of them are subjected to metabolic transformations incident to their becoming physiologically established and thereby acquire a new chemical character;—these are only a few of the fundamental questions that suggest themselves.

In the past the numerous investigations of fat formation have usually been complicated by dietary variables. Change from one

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ration to another has frequently involved alterations, sometimes unsuspected, in several components of the food intake. The development of the science of nutrition has more recently made it possible to plan experimental feeding under conditions in which a single factor can be altered at will without profoundly upsetting the proportionate relations of the rest of the diet. Accordingly we have planned to supply certain indispensable food ingredients in constant proportions to animals—to furnish protein, inorganic salts, vitamins, and additional sources of energy under uniform conditions—while varying the possible precursors for fat formation.

Plan of Experiments.

The feeding experiments were conducted with young albino rats. Their diets were planned as a rule to supply 40 per cent of the total calorie intake in the form of dried skimmed milk. This quantity was selected to furnish sufficient protein of suitable quality for the needs of the growing animal, as well as an adequate mixture of inorganic salts. Some vitamin B was thus supplied, but yeast and cod liver oil were added in fixed daily quantities to assure an adequate intake of the indispensable vitamins. The remaining 60 per cent of the calorie intake was usually provided in the form of fats or carbohydrates.

In a few instances other rations were employed. One of these consisted of a mixture of whole milk powder and ground whole wheat. A few rats also were fed proteins (casein and cottonseed globulin) at very high levels of intake. The animals were killed at various body weights and the fat was rendered for analysis under special conditions to avoid decomposition[1] as far as possible.

Details of Technique Employed.

The Animals and Their Care.—Male albino rats which weighed between 50 and 55 gm. at the beginning of the feeding tests were kept individually in cylindrical wire mesh cages (Ferry, 1919–20) and were weighed twice weekly.

[1] As a measure of hydrolysis, free fatty acid determinations were made on a number of samples and were found to range from 7 to 9.5 per cent (calculated as oleic acid).

Method of Obtaining Body Fat.—Immediately after the animals were killed with illuminating gas the stomach and the intestines were removed in order to exclude any food fat; all of the remaining tissue was comminuted in a meat grinder. The hash was then transferred to a 1 liter Erlenmeyer flask containing 300 to 400 cc. of distilled water; the flask was covered with an inverted beaker and placed in an autoclave. In the latter the animal fat was liberated by being rendered in an atmosphere of steam at a pressure of 38 to 40 pounds for a period of 14 or 15 hours. The autoclave was immersed in a bath of heavy cylinder oil, electrically heated with attached space heaters. A motor-operated switch with thermostat (Tycos Temperature Control) facilitated the automatic maintenance of the pressure within the range mentioned. The oil in the oil bath was well stirred by an electrically driven paddle shortly after heating operations were started.

At the completion of the rendering process (and after the autoclave had cooled off sufficiently to open it without danger of loss of rendered material) the liquid contents containing the melted fat or oil were decanted into a separatory funnel. The residue was washed several times with hot water, the washings also being transferred to the separatory funnel. After a careful separation of the crude fat from the aqueous layer was effected, the fat was filtered in an air oven at about 60°.

Specimens of fat so procured were usually kept in amber jars in a refrigerator until analyzed. To prevent as far as possible the oxidation of the fats the specimens secured more recently were preserved in well stoppered vials selected in each case so as to be nearly filled with the available fat.

Analytical Values.—Inasmuch as we were primarily interested at the outset in acquiring a knowledge of the degree of unsaturation of the fatty acids present in the fat produced on various diets, we determined the iodine value by the Hanus method. All analyses were made in duplicate. In a number of cases we also determined the refractive indices.

Character of Body Fat Produced by a Fixed Diet at Various Stages of the Animal's Development.

As a guide to changes in the character of the body fat that might be due to more purely physiological, in contrast with dietary factors, the body fat present in animals representing different stages

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in development and ranging in body weight from 120 to 330 gm. was examined. The food mixture, unchanged throughout the course of these tests, consisted of one-third dried whole milk and two-thirds ground whole wheat.[2] The results recorded in Table I show that the body fat tends to become progressively "harder" with the increasing weight (advancing age) of the animal. Apparently the rat and the hog (Ellis and Hankins, 1925) behave alike in that with increasing maturity both species tend to produce

TABLE I.

Diet: Ground whole wheat (2 parts), dried whole milk (1 part).

Rat No.	Final body weight.	Body fat.	
		Iodine No.	Refractive index at 40°.
	gm.		
1134	120	66.7	1.4608
1136	120	61.1	1.4599
1135	136	60.9	1.4596
1137	150	61.1	1.4603
1100	175	58.3	1.4598
1101	200	62.8	1.4600
1081	246	56.2	1.4594
1083	272	53.6	1.4595
1082	310	55.6	1.4593
1085	330	58.3	1.4596

"harder" fats. On the other hand, Moulton and Trowbridge (1909) found that in the beef animal "the iodine value of fat from the fatty tissue of an animal increases [*i.e.* grows softer] with the age of the animal." Of course these comparative statements are based upon results obtained with unlike experimental rations for different species.

Selection of Suitable Experimental Diets.

Experience has shown that satisfactory growth in rats may be secured with diets having a protein content of 15 per cent of the total calorie intake provided that the protein is of satisfactory

[2] This diet with the addition of sodium chloride in the proportion of 2 per cent of the weight of wheat, has been used extensively by H. C. Sherman and his coworkers. See Sherman, H. C., and Muhlfeld, M., *J. Biol. Chem.*, 1922, liii, 41.

biological quality (Osborne and Mendel, 1916). This is, however, not the optimal content.

When dried skimmed milk is fed in proportions sufficient to supply protein calories at a 15 per cent level, the additional non-protein nutrients therein make the energy contribution of the milk equivalent to 40 per cent of the total calorie intake. The remaining 60 per cent of the calorie requirement can then be supplied in the form of fats, carbohydrates, or mixtures thereof. The nutritive effectiveness of such mixtures obviously depends on a satisfactory total food consumption on the part of the animals fed. Of this their vigorous growth has been taken as sufficient evidence.

Table II shows the approximate composition[3] of the dried skimmed milk (Klim brand) used.

In calculating the desired calorie adjustments, the conventional values: protein 4.1, carbohydrate 4.1, fat 9.3 calories per gm. were employed. A simple calculation shows that 1 calorie from protein is supplied by 0.244 gm. of protein represented by 0.726 gm. of dried skimmed milk. 15 calories of protein origin would therefore require 10.89 gm. of dried skimmed milk which supplies a total of 40 calories.

The following illustrative computations indicate the application of the principles described:

Diet, Type A, Rich in Fat.

	Weight.	Energy value.	Total calories.
	gm.	*calories*	*per cent*
Fat	37.2	346	60
Dried skimmed milk	62.8	230	40
Total	100.0	576	100

Diet, Type B, Rich in Carbohydrate.

	Weight.	Energy value.	Total calories.
	gm.	*calories*	*per cent*
Carbohydrate	57.3	235	60
Dried skimmed milk	42.7	156	40
Total	100.0	391	100

[3] An analysis of dried skimmed milk (Klim brand) was found in a bulletin published by the Connecticut Agricultural Experiment Station (*Connecticut Agric. Exp. Station, Bull. 227*, 1920, 253).

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Influence of Various Foodstuffs on Body Fats.

1. Fats.—The food fats selected included soy bean oil, corn oil, cottonseed oil (winter-pressed), peanut oil, Crisco (a hydrogenated vegetable oil), lard, butter fat, and coconut oil, thus affording comparisons of wide variations in their fatty acid make-up.

The results of the feeding experiments are detailed in Table III, and the outcome is summarized to permit a ready comparison of the relations of the composition of the food fat to the body fat (Table IV).

It will be observed in several instances that the composition of the deposited tissue fat, in so far as this is indicated by the iodine values, resembles quite closely that of the ingested fat. This is notably true for soy bean oil, corn oil, cottonseed oil, peanut oil,

TABLE II.

Approximate Composition of Dried Skimmed Milk (Klim Brand).

Dried skimmed milk.	Ingredients.	Calories per kilo.
	per cent	
Moisture	4.0	
Ash	8.2	
Protein	33.6	1378
Fat	1.1	102
Sugar	53.1	2177
Total	100.0	3657

and also Crisco. Contrast, on the other hand, the animal fats produced when, for example, butter fat or coconut oil is consumed. Apparently the animal alters these dietary fats by a desaturating process. Lard was also desaturated to some extent.

These findings demonstrate the profound effect of liberal intake of fats upon the quality of the body fat and serve to emphasize a feature of fat metabolism not always understood. Thus Dr. H. W. Wiley (1926) has said: "All the fats we eat are burned in the body. The fats that are deposited in our bodies are made from sugar and starch and not from the fats we eat." Our results are not in accord with his statement.

Attention should be called to a small error incident to the method of fat rendering employed in its application to the results with fats relatively rich in glycerides of the volatile fatty acids (*e.g.*, butter fat and coconut oil).

TABLE III.

Iodine and Refractive Index Values of Body Fats Produced when Various Food Fats Were Ingested.

Diet.

Fat.....60 per cent calories.
Dried skimmed milk.....40 " " "

Food fat, soy bean oil.				Food fat, corn oil.			
		Body fat.				Body fat.	
Rat No.	Final body weight.	Iodine No.	Refractive index at:	Rat No.	Final body weight.	Iodine No.	Refractive index at:
			25°				25°
	gm.				gm.		
300	132	121.4	1.4768	1039	136	115.8	1.4727
1037	134	122.2	1.4749	1122	136	111.2	1.4713
294	156	122.9	1.4729	290	154	114.1	1.4724
299	160	123.5	1.4731	289	160	114.6	1.4724
1034	240	122.7	1.4730	1040	292	113.2	1.4726
301	250	122.7	1.4728	309	320	116.1	1.4723
Soy bean oil.....		132.3	1.4736	Corn oil.....		124.2	1.4726
Food fat, cottonseed oil.				Food fat, peanut oil.			
			25°				25°
1118	140	103.4	1.4707	1121	135	93.5	1.4700
291	154	108.5	1.4714	1119	140	94.2	1.4701
292	160	109.6	1.4719	274	155	100.9	1.4700
260	254	108.2	1.4713	270	160	99.8	1.4701
1042	260	108.7	1.4713	271	163	100.8	1.4703
1032	308	105.7	1.4708	278	260	100.0	1.4699
Cottonseed oil.....		108.1	1.4713	276	296	100.0	1.4702
				Peanut oil.....		102.4	1.4702
Food fat, Crisco.				Food fat, lard.			
			40°				40°
1128	160	82.0	1.4626	1132	164	73.4	1.4618
1129	172	81.3	1.4625	1133	170	73.7	1.4620
1046	282	82.5	1.4623	1048	190	68.6	1.4621
1045	322	81.6	1.4618	1047	318	71.1	1.4613
Crisco.....		78.8	1.4626	Lard.....		63.2	1.4603
Food fat, butter fat.				Food fat, coconut oil.			
			40°				25°
1130	152	55.2	1.4598	1094	148	37.2	1.4608
1131	158	59.4	1.4603	1095	150	34.6	1.4608
1043	216	52.4	1.4592	1096	150	34.1	1.4607
1044	294	55.0	1.4591	Coconut oil.....		7.7	1.4557
Butter fat.....		35.8	1.4550				

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The latter acids may be in part lost through the autoclave procedure. Consequently, the iodine values representing unsaturated fatty acids may be relatively higher for the body fats as analyzed than the actual undecomposed tissue might represent.

TABLE IV.

Summary of Iodine Values Contained in Table III.

Food fat (60 per cent of total calories).	Iodine No. of food fat.	Iodine No. of body fat (averages).
Soy bean oil	132.3	122.5
Corn oil	124.3	114.2
Cottonseed oil	108.1	107.4
Peanut oil	102.4	98.4
Crisco	78.8	81.8
Lard	63.2	71.7
Butter fat	35.8	55.5
Coconut oil	7.7	35.3

TABLE V.

Diets.	Rat No.	Final body weight.	Body fat.	
			Iodine No.	Refractive index at 40°.
		gm.		
Starch 60 per cent calories. Dried skimmed milk 40 " " "	308	132	57.7	1.4600
	307	142	63.6	1.4598
	1145	152	58.3	1.4600
	304	168	60.3	1.4600
	302	220	59.8	1.4595
	303	250	59.2	1.4602
	306	256	61.3	1.4596
	305	280	61.5	1.4598
Dextrin 60 per cent calories. Dried skimmed milk 40 " " "	1153	164	57.8	1.4599
	1154	178	58.3	1.4601
Dextri-Maltose 60 per cent calories. Dried skimmed milk 40 " " "	1161	145	55.0	1.4591
	1160	156	58.3	1.4599

2. Carbohydrates.—When corn-starch (diet, Type B) was substituted equicalorically for fat in the ration, an animal fat was produced which had an iodine value of about 60 (Table V).

Regarding the origin of this hard body fat, it is necessary to

consider the fat content of the various food components employed in this experimental series. The dried skimmed milk contained 1.1 per cent fat (Table II), and according to Taylor and his collaborators (1920, 1926), the starch of the sort used presumably contains small quantities (approximately 0.7 per cent) of associated and combined fat. These two items supplied at most only about 2 per cent of the total calories of the diet in the form of fat. The cod liver oil and yeast supplements furnished additional small sources. Although these various small fat inclusions seem in-

TABLE VI.

Composition of Casein and Cottonseed Globulin.

	Casein.			Cottonseed globulin.		
	Weight.	Energy value.	Total calories.	Weight.	Energy value.	Total calories.
	gm.	*calories*	*per cent*	*gm.*	*calories*	*per cent*
Moisture	9.28			8.60		
Ash	2.80			1.14		
Protein	85.81	352	96.4	87.55	359	96.2
Ether extract	0.87	8	2.2	0.57	5	1.3
Nitrogen-free extract	1.24	5	1.4	2.14	9	2.5
Total	100.00	365	100.0	100.00	373	100.0

To convert nitrogen to protein, the factors 6.38 and 5.7 were used for casein and cottonseed globulin respectively.

significant in relation to the total food intake, we have estimated that if they were absorbed and stored without loss in the body, they might in the course of the entire feeding period be sufficient to account for practically all the fat present in the body at the end of the experimental period. Despite this mathematical possibility it seems unlikely, however, that the tissue fat at the end of the feeding test was actually attributable to such selective storage without any destruction of the fat consumed in small quantities from day to day, rather than to any synthesis whatever from the large amounts of carbohydrate ingested.

In addition to corn-starch, two other carbohydrates—dextrin and Dextri-Maltose[4]—were fed to small series of animals. All three series produced a similar quality of animal fat (see Table V).

[4] Dextri-Maltose No. 2, the sort used, contains maltose 52 per cent, dextrin 43 per cent, and moisture 5 per cent.

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3. Proteins.—Casein and cottonseed globulin, analyses of which are shown in Table VI, were each fed at the same very high concentration; namely, 95 per cent by weight (Osborne and Mendel, 1924). The other components of the diet were Osborne and Mendel's salt mixture[5] 5 per cent and the usual vitamin-bearing supplements. The results recorded in Table VII show that the fats produced on diets very high in protein concentration are similar in quality, as far as iodine values may be a criterion, to fats produced on diets rich in carbohydrate (Table V). This is what might be expected in view of the recognized conversion of the non-nitrogenous portions of the protein molecule into carbohydrate under suitable conditions of metabolism (Atkinson, Rapport, and Lusk, 1922).

TABLE VII.

Diets containing:	Rat No.	Final body weight.	Iodine No.	Body fat refractive index at 40°.
		gm.		
Casein 95 per cent.	1080	158	64.1	1.4628
	1126	162	68.0	1.4607
	1079	178	63.0	1.4618
Cottonseed globulin 95 per cent.	1318	175	68.6	
	1199	190	69.6	
	1202	210	66.6	
Casein 80 per cent; starch 15 per cent.	1076	190	67.3	1.4607
	1077	208	62.7	1.4604
	1078	254	65.9	1.4604

It should be added, however, that despite the small fat content, 0.87 per cent and 0.57 per cent, of the casein and cottonseed globulin respectively, we find by estimation as we did with the rich-in-carbohydrate series that in some instances the fat supplied in the daily supplements of cod liver oil and yeast might be sufficient in itself to account for the quantity of fat in the animal body when the analysis was made.

In addition to the groups of rats above referred to, which re-

[5] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

ceived the proteins, casein, and cottonseed globulin, a series was fed a mixture of casein and starch. The composition of the ration was as follows: casein 80 per cent, starch 15 per cent, and salts 5 per cent, and the usual vitamin-bearing supplements in addition.

The body fats produced on this ration were of the same "hardness" as that secured when either casein or cottonseed globulin were fed in very high concentration (Table VII).

TABLE VIII.

"Hardening" Effect of a Diet Rich in Carbohydrate (Corn-Starch) upon the Body Fat Produced by Dietary Fats.

The energy intake of the diets in terms of total calories was distributed as follows:

	Preliminary (milk + fat) period. *per cent*	"Hardening" (milk + starch) period. *per cent*
Dried skimmed milk	40	40
Fat	60	
Starch		60

Diets.	Rat No.	Final body weight on milk + fat diet.	Final body weight on milk + starch diet.	Iodine No. of body fat.	Duration of milk + starch feeding.
		gm.	*gm.*		*days*
Milk + *soy bean oil* followed by milk + starch.	1098	135	208	77.5	29
	295	136	240	65.4	87
	297	140	248	64.7	81
	293	134	262	63.6	96
Milk + *peanut oil* followed by milk + starch.	1543	148	238	78.8	35
	1535	150	240	73.4	49
	1548	150	274	72.4	55
	1570	152	292	65.6	68

Effect of Dietary Changes after Fats of Definite Type Have Been Deposited in the Body.

Rats were raised to weights between 134 and 140 gm. on rich-in-fat diets (Type A) containing soy bean oil (Table VIII). Another group was similarly raised to 150 gm. (± 2.0 gm.) on the peanut oil ration. The food mixture was then changed to the carbohydrate-rich diet (Type B) with the result that a progressive "harden-

ing" of the body fat ensued. For example, rat fat with an expected iodine value of approximately 123, produced on the soy bean oil diet, was changed by subsequent starch feeding to a quality represented by iodine numbers ranging from 77 to 63. The greater the weight attained on the carbohydrate diet, the "harder" was the fat obtained, and correspondingly the lower was its iodine value.

Depletion of Fat Reserves as a Method of Promoting More Rapid Subsequent Dietary Effects upon Body Fat.[6]

In view of the slow progressive "hardening" of "soft" fat by a change to a diet rich in carbohydrate there naturally arose the question of the possible effect of fat depletion prior to the shifting to the "hardening" carbohydrate ration. The fat reserves can be depleted by fasting. The researches of Lebedeff (1882) and likewise of Munk (1884) suggest this method. These investigators studied the effects of feeding foreign fats to dogs subsequent to a period of fasting. Accordingly we subjected rats, grown to various weight levels on oil-containing diets to the fasting treatment before feeding the "hardening" diet. Furthermore, for comparison we raised rats under precisely the same conditions, including a change to the "hardening" ration, without any preliminary fat-depleting process at the end of the oil-feeding régime.

During the period of so called fasting, not only was water given *ad libitum*, but the vitamin-bearing materials, cod liver oil and dried yeast, were supplied to all except a few rats,[7] our expectation being that the maintenance of the body stores of vitamins would promote a more rapid growth recovery upon realimentation.

The comparative data obtained from fasted and non-fasted groups of rats originally grown on soy bean oil, peanut oil, and corn oil to varying weights, are recorded in Tables IX to XI, respectively.

The losses in body weight experienced by rats subjected to the

[6] A preliminary report of this phase of our work has been published by Mendel and Anderson (Mendel, L. B., and Anderson, W. E., *Science*, 1926, lxiv, 384). This article was copied in *J. Oil and Fat Ind.*, 1926, iii, 396.

[7] The few exceptions that were not given cod liver oil and yeast while being fasted were Rats 1401, 1402, 1404, 1405, and 1406, all of which were raised originally on the diet containing corn oil (Table XI).

TABLE IX.

"Hardening" Effect of a Diet Rich in Carbohydrate (Corn-Starch) upon the Body Fat Produced by Soy Bean Oil.

Comparison of Effects of Fasting (Resulting in Depletion of Fat Reserves) and of Non-Fasting Prior to Substitution of Carbohydrate in Ration.

The energy intake of the diets in terms of total calories was distributed as follows:

	Preliminary (milk + fat) period. *per cent*	"Hardening" (milk + starch) period. *per cent*
Dried skimmed milk	40	40
Soy bean oil	60	
Starch		60

	Rat No.	Final body weight on milk + fat diet.	Body weight after fasting.	Loss by fasting.	Final body weight on milk + starch diet.	Iodine No. of body fat.	Milk + starch diet consumed.	Duration of milk + starch feeding.
		gm.	*gm.*	*per cent*	*gm.*		*gm.*	*days*
Rats raised to *150 gm.* (±3.0 gm.) on milk + fat diet.	1450	150	107	28.7	198	67.8	301	20
	1449	152	108	29.0	198	63.9	203	14
	1452	153	113	26.1	197	64.4	211	15
	1563	150	102	32.0	196	64.2	268	26
	1550	150	102	32.0	196	68.9	321	25
	1552	150	95	36.7	195	64.4	254	19
	1457	148	Non-fasted.		197	97.7	188	13
	1453	152	"		195	93.8	199	14
	1455	150	"		198	83.5	233	14
	1561	150	"		198	99.9	226	19
	1564	152	"		200	101.9	158	13
Rats raised to *225 gm.* (±3.0 gm.) on milk + fat diet.	1445	224	165	26.3	258	73.3	697	49
	1458	226	169	25.2	266	81.4	529	35
	1558	223	150	32.7	270	72.9	484	35
	1556	222	144	35.1	264	62.8	429	28
	1460	225	Non-fasted.		268	109.7	531	34
	1461	224	"		268	103.4	483	33
	1447	223	"		268	103.7	497	36
Rats raised to *250 gm.* (±3.0 gm.) on milk + fat diet.	1456	248	184	25.8	275	71.4	453	27
	1463	248	182	26.6	280	76.0	533	35
	1464	247	185	25.1	272	71.5	435	27
	1559	252	170	32.5	272	72.1	465	31
	1448	251	Non-fasted.		280	115.5	442	31
	1454	250	"		288	108.5	355	26
	1462	249	"		268	109.9	406	28
	1555	248	"		280	114.2	272	20

TABLE X.

"Hardening" Effect of a Diet Rich in Carbohydrate (Corn-Starch) upon the Body Fat Produced by Peanut Oil.

Comparison of the Effects of Fasting (Resulting in Depletion of Fat Reserves) and of Non-Fasting Prior to Substitution of Carbohydrate in Ration.

The energy intake of the diets in terms of total calories was distributed as follows:

	Preliminary (milk + fat) period. *per cent*	"Hardening" (milk + starch) period. *per cent*
Dried skimmed milk	40	40
Peanut oil	60	
Starch		60

	Rat No.	Final body weight on milk + fat diet.	Body weight after fasting.	Loss by fasting.	Final body weight on milk + starch diet.	Iodine No. of body fat.	Milk + starch diet consumed.	Duration of milk + starch feeding.
		gm.	*gm.*	*per cent*	*gm.*		*gm.*	*days*
Rats raised to *150 gm.* (±3.0 gm.) on milk + fat diet.	1508	148	113	23.7	185	67.5	161	17
	1511	147	110	25.2	190	65.8	220	14
	1537	152	100	34.2	183	56.3	195	17
	1565	150	94	37.3	183	55.1	211	18
	1506	149	Non-fasted.		182	81.4	207	13
	1514	147	"		184	83.1	212	17
	1542	152	"		184	81.6	200	16
	1544	153	"		188	93.5	155	14
Rats raised to *175 gm.* (±3.0 gm.) on milk + fat diet.	1488	172	126	26.7	228	61.9	301	20
	1479	175	128	26.9	223	63.5	339	21
	1487	175	134	23.4	221	68.0	365	24
	1536	174	115	33.9	224	63.6	489	32
	1538	175	115	34.3	222	64.8	381	31
	1478	177	Non-fasted.		225	78.3	372	21
	1490	173	"		224	75.0	345	24
	1492	175	"		225	74.3	314	24
	1566	172	"		223	74.5	553	41
	1569	175	"		230	83.5	354	23
Rats raised to *225 gm.* (±3.0 gm.) on milk + fat diet.	1480	224	170	24.1	270	71.3	470	29
	1491	222	160	27.9	248	66.8	640	46
	1567	228	152	33.3	265	64.3	450	31
	1485	225	Non-fasted.		260	87.9	594	46
	1481	225	"		268	82.9	400	28
	1539	225	"		266	84.0	621	48

TABLE X—*Concluded.*

	Rat No.	Final body weight on milk + fat diet.	Body weight after fasting.	Loss by fasting.	Final body weight on milk + starch diet.	Iodine No. of body fat.	Milk + starch diet consumed.	Duration of milk + starch feeding.
		gm.	*gm.*	*per cent*	*gm.*		*gm.*	*days*
Rats raised to *250 gm.* (±3.0 gm.) on milk + fat diet.	1475	247	186	24.7	275	69.5	535	33
	1484	247	175	29.2	275	60.0	325	17
	1568	248	158	36.3	278	62.3	372	24
	1476	248	Non-fasted.		282	87.2	588	52
	1482	250	"		272	91.1	491	33
	1483	249	"		282	86.8	462	32

fasting treatment ranged from 18.8 to 40.6 per cent, most losses being between 22 and 35 per cent. As was anticipated, the fats yielded by fasted groups of rats were in every case appreciably "harder;" *i.e.*, of lower iodine value than those obtained from the corresponding non-fasted groups. For example, the ranges in iodine values of fats produced by the fasted and non-fasted groups of rats, originally raised to 175 gm. on the peanut oil diet, respectively were 61.9 to 68.0 against 74.3 to 83.5. However, the most striking differences in "hardness" of fats yielded by fasted and non-fasted groups of rats appear in animals first raised to fully mature weights on oily diets. Thus, the ranges of the iodine values of fats produced in rats raised to 250 gm. on soy bean oil diet, by fasted and non-fasted groups, were 71.4 to 76.0 and 108.5 to 115.5, respectively. Such results show quite clearly that the preparatory depletion of the fat reserves does promote more rapid subsequent dietary effects upon body fat.

Inasmuch as the relative economy of animal production under conditions involving temporary fat depletion is a matter of large interest, we obtained data on the duration of the feeding period and the amount of the milk and starch diet consumed for both fasted and non-fasted groups. These data are included in Tables IX to XI, already referred to.

It might be assumed that losses in body weight amounting to

TABLE XI.

"Hardening" Effect of a Diet Rich in Carbohydrate (Corn-Starch) upon the Body Fat Produced by Corn Oil.

Comparison of Effects of Fasting (Resulting in Depletion of Fat Reserves) and of Non-Fasting Prior to Substitution of Carbohydrate in Ration.

The energy intake of the diets in terms of total calories was distributed as follows:

	Preliminary (milk + fat) period. *per cent*	"Hardening" (milk + starch) period. *per cent*
Dried skimmed milk	40	40
Corn oil	60	
Starch		60

	Rat No.	Final body weight on milk + fat diet.	Body weight after fasting.	Loss by fasting.	Final body weight on milk + starch diet.	Iodine No. of body fat.	Milk + starch diet consumed.	Duration of milk + starch feeding.
		gm.	*gm.*	*per cent*	*gm.*		*gm.*	*days*
Rats raised to *150 gm.* (±3.0 gm.) on milk + fat diet.	1465	149	116	22.2	200	73.1	216	16
	1471	152	114	25.0	196	67.1	255	19
	1401	150	121	19.3	200	74.6	195	14
	1402	149	121	18.8	206	70.3	215	19
	1472	153	Non-fasted.		200	91.5	224	17
	1467	150	"		202	86.5	233	18
	1412	149	"		197	94.4	198	14
	1419	150	"		200	81.8	259	17
Rats raised to *175 gm.* (±3.0 gm.) on milk + fat diet.	1404	175	135	22.9	222	69.4	220	14
	1405	172	138	19.8	230	70.1	292	18
	1406	175	140	20.0	232	79.6	292	17
	1576	175	104	40.6	222	68.6	295	23
	1573	174	Non-fasted.		230	88.0	348	29
	1574	177	"		227	104.0	332	23
	1575	176	"		226	*	386	25
Rats raised to *250 gm.* (±3.0 gm.) on milk + fat diet.	1424	253	186	26.8	270	70.9	561	38
	1425	251	188	25.1	280	72.7	390	24
	1580	250	160	36.0	278	70.3	406	28
	1579	248	162	34.7	278	63.5	315	21
	1473	248	Non-fasted.		280	108.5	521	38
	1432	252	"		280	114.0	263	16
	1578	248	"		278	*	416	29

* Fat not recovered.

25 per cent or more would call for a very much larger supply of energy to produce the same final (killing) weight attained by rats not subjected to the depletion treatment. Our experiments show the food intake[8] and energy consumption of the rats realimented after considerable loss in body weight to be in most cases not materially larger than (in some cases approximately equal to) the intakes of animals attaining the same body weights[9] without interruption of growth.

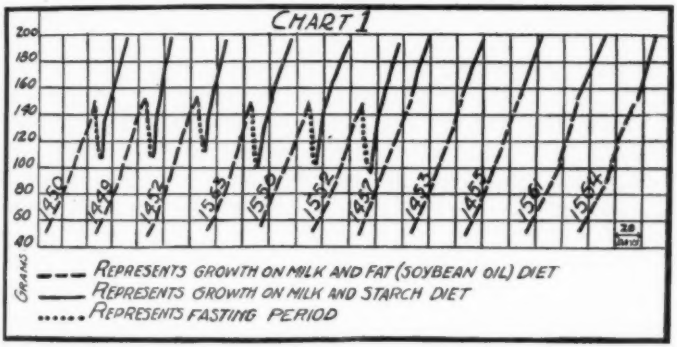

CHART 1.

Chart 1 which is typical of others which might be presented includes the growth curves of groups of rats originally raised to 150 gm. on the soy bean oil ration. The very rapid recovery growth experienced by the fasted group is readily visualized.

SUMMARY.

The technique of obtaining the fat of the entire animal for analysis is described. The iodine numbers and refractive indices

[8] In an effort to obtain as far as possible an accurate measure of the food intake, the dry food mixture spilled on the screen and paper on the bottom of each cage was collected and returned daily to the food jar.

[9] It was planned to have rats of both fasted and non-fasted groups grow on the milk and starch diet until they had attained approximately the same final body weights. In a few cases the rats failed to grow beyond the weights recorded in the columns marked "final body weight on milk + starch diet" in Tables IX to XI.

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of the body fat produced in rats on a variety of specially planned dietary régimes were determined.

In order to ascertain the possible changes in the character of the fat deposited at various stages of development, observations were made on animals receiving a uniform mixture of whole wheat and whole milk. On this ration the body fat tends to have a lower iodine value; *i.e.*, becomes "harder" as the body weight increases.

A scheme of experimental feeding was further devised by which the animals received 15 per cent of their calorie intake in the form of protein. This was supplied by dried skimmed milk in amounts to furnish 40 per cent of the total calories of the ration. The remaining 60 per cent of the energy intake was variously offered in the form of fat or carbohydrate. In this way it was possible to maintain a constant proportion of the essentials of the diet while the nutrients available for fat formation could be varied at will. Striking differences were observed when different types of food were thus made to constitute the variable 60 per cent of the calorie intake. When soy bean oil, corn oil, cottonseed oil, or peanut oil was ingested the resulting body fat resembled the food fat. On the other hand, the body fats produced when butter fat or coconut oil was consumed contained considerably more unsaturated fatty acids than the ingested fat. The outcome of this whole series of feeding tests is summarized in Table IV. When the carbohydrates, corn-starch, dextrin, and Dextri-Maltose were substituted equicalorically for fat in the diet, "hard" body fats with iodine values of about 60 were obtained. This "harder" type of fat presumably included a relatively higher proportion of saturated fatty acids. When proteins (casein and cottonseed globulin) were fed in exceptionally high concentration—amounting to 96 per cent of the total calories—a "harder" fat was produced, approximating in its iodine value the fat of the rats receiving the diets rich in carbohydrate.

When a particular type of "soft" body fat has been developed, its chemical make-up can be altered by a change to the "hardening" ration rich in carbohydrate. Such alterations which presumably involved a partial depletion or transformation of the fat reserves are accomplished only slowly. The change from one type of specially produced depot fat to another can be brought about far more rapidly by depleting the fat reserves through brief periods

of fasting prior to the change in the dietary. For example, when the fat-producing components of a ration were changed from 60 calories per cent of peanut oil to equicaloric proportions of carbohydrate, the iodine value at the end of 68 days of feeding was not lower than 65 (Table VIII); whereas, when a preliminary loss of the fat reserves was brought about by withholding food, the subsequent feeding of the carbohydrate diet both restored the animals to the size of companion non-fasted rats and led to the deposition of an even "harder fat"—iodine number 55—in the comparatively short space of 18 days (Table X).

We desire to thank Procter and Gamble Company for liberal quantities of soy bean oil, peanut oil, and coconut oil; Dr. G. S. Jamieson, Bureau of Chemistry, United States Department of Agriculture, for samples of soy bean oil and peanut oil used in preliminary experiments; and Mead Johnson and Company for a supply of Dextri-Maltose No. 2.

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THE INFLUENCE OF SODIUM FLUORIDE AND THYMOL ON THE DETERMINATION OF UREA BY THE UREASE METHOD.

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(Received for publication, November 18, 1927.)

Erroneous results obtained in this laboratory for the urea values of human bloods which had been preserved by means of sodium fluoride and thymol according to the method of Sander (7), have led us to investigate whether these preserving substances interfere with the estimation of urea by the urease method. Sander in his study employed the Folin-Wu technique for the estimation of urea but states that some of the later analyses were conducted on whole blood. While the present work was in progress a short paper by Kildruffe and Springer (4) appeared, calling attention to the fact that in a series of twenty cases the urea nitrogen values determined by the Folin and Wu technique were found to be considerably lower when sodium fluoride and thymol were added to the blood. The decrease in concentration of urea showed no regular effect. We have investigated the question more extensively and have also come to the conclusion that urea values obtained by the urease method on whole blood, according to the method of Cullen and Van Slyke, in the presence of sodium fluoride and thymol are unreliable. Several authors have asserted that the mixture proposed by Sander is satisfactory for the preservation of blood in which the amounts of the nitrogenous as well as the non-nitrogenous products are to be determined. Denis and Beven (2), using the Folin-Wu method, showed in a series of analyses of the blood of man and of dogs that, when a 10:1 mixture of sodium fluoride and thymol was added in a concentration of 6 mg. for each cc. of blood, either with or without potassium oxalate, the values for urea nitrogen were constant; but in none of their cases did the value of urea nitrogen exceed 26 mg. for each 100 cc. The significance of this will be pointed out in the experimental part of this paper. Major (5) called attention to the fact that glycolysis was inhibited by the presence of sodium fluoride and that reliable values for sugar and carbon dioxide could be obtained in bloods preserved for at least 1 week. John (3) also came to this conclusion, but neither of these authors gave any experimental data for the estimation of urea. Cameron and Williamson (1) state that the values for urea are constant for varying periods of time, and their figures bear out their statement that normal values for urea were constant over a

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period of 10 days. They only cite, however, analysis of three bloods from pathologic cases in which the urea value was higher than normal; the highest was 69 mg. for each 100 cc. of blood. This value remained constant for 3 days. Schwentker (8) states that sodium fluoride is an efficient preservative in the analysis of blood for nitrogenous elements as well as for sugar. He states that his results corroborate the statements of Sander for nitrogenous elements and also those of John, and Denis and Beven, but gives no experimental results to show whether his analyses were conducted on normal bloods or pathologic bloods in which a high content of urea might be expected.

Since this paper was submitted for publication, an article by Roe, Irish, and Boyd (6) appeared in which it is shown that urea cannot be quantitatively estimated by Myers' technique in aqueous solutions or in blood containing sodium fluoride unless the concentration of sodium fluoride is decreased to approximately 3 mg. in each cc. The blood was diluted with water or the fluoride precipitated by magnesium salts previous to hydrolysis with urease. In water solutions urease will tolerate a concentration of sodium fluoride of less than 1 mg. in each cc., whereas blood will tolerate a concentration of 3.75 mg. in each cc. without inhibition of the activity of urease. This concentration is considerably greater than that of a Folin-Wu filtrate obtained from blood preserved by sodium fluoride in a concentration of 10 mg. in each cc. From this work it would appear that the concentration of sodium fluoride present in a Folin-Wu filtrate should not interfere with urease activity. This is questioned, however, by the data of Kildruffe and Springer (4).

Our decision to investigate the action of a mixture of sodium fluoride and thymol on the determination of urea by the urease method was prompted by the discovery that the urea values of bloods removed post mortem invariably analyzed low. This was contrary to our expectations, especially when we found that the antemortem urea values of unpreserved blood were higher than the values determined post mortem on preserved blood. These bloods had been preserved by the sodium fluoride and thymol mixture of Sander (7).

The effect of sodium fluoride and thymol mixture on the estimation of urea by the urease method on whole blood is illustrated in the discussion of the tables. 10 mg. of the preservative were used for each cc. of blood unless otherwise stated. We have been unable to confirm the statement of Sander that 10 times this quantity could be used without deleterious effect. 100 mg. of Arlco urease for each estimation and an incubation period of 30 minutes with subsequent aeration constituted the routine unless otherwise stated.

In Table I is shown the effect of sodium fluoride and thymol mixture on the urea concentration of bloods which have a urea value of not more than 46 mg. for each 100 cc. of blood. Urea was then determined in the same bloods after the addition of 1 cc. of a urea solution. The urea solution referred to in Table I gave

TABLE I.

Effect of Sodium Fluoride and Thymol Preservative on Urea Value When Added to Solutions of Urea and to Blood.

Blood No.	1	2	3	4	5	6	7	8	9
	Mg. urea per 100 cc. blood.								
Blood + potassium oxalate	34	46	28	26	22	24	32	36	30
" + sodium fluoride and thymol	28	32	20	18	12	12	14	16	8
" + 1 cc. urea solution + potassium oxalate	156	168	152	154	146	150	152	162	156
Blood + 1 cc. urea solution + sodium fluoride and thymol	62	78	44	69	66	46	38	28	30

TABLE II.

Effect of Variable Amounts of Sodium Fluoride and Thymol Preservative on Urea Values When Added to Aqueous Urea Solutions and to Blood.

Sodium fluoride + thymol, mg. per cc.	Urea.			Blood 1.	Blood 2.
	Solution 1.	Solution 2.	Solution 3.		
	Mg. urea per 100 cc.				
None.	132	126	128	312	60
10					58
20					44
30	28	62, 80, 44	60, 50, 102	93	34
40				69	26
50	18			72	38
80	8				
100	4				

a urea value in the presence or absence of potassium oxalate of 126 mg. in each 100 cc. The urea value, after the addition of the sodium fluoride and thymol mixture with and without the addition of potassium oxalate varied in the former instance from 32 to 80 mg. and in the latter from 50 to 102 mg. in each 100 cc.

It is clear from Table I that the effect of sodium fluoride is to lower markedly the value of the urea concentration; the effect being greater as the urea concentration increases. The same effect is

TABLE III.

Effect of Sodium Fluoride and Thymol on Urea Value of Oxalated Blood.

Sample No.	3 mg. potassium oxalate per cc.	3 mg. sodium fluoride per cc.
	Mg. urea per 100 cc. blood.	
Blood.		
1	22	22
2	22	22
3	24	22
4	28	30
5	32	30
6	38	38
7	46	44
8	44	34
9	52	48
10	198	120
		10 mg. sodium fluoride per cc.
11	26	24
12	30	30
13	38	36
14	54	48
15	62	58
16	70	66
17	126	48
18	148	118
19	156	62
20	312	72
Blood + urea solution.		
21	213	56
22	156	62
23	158	78
24	142	44
25	154	69
26	146	66
27	140	46
28	152	38
29	162	28
30	156	30

shown also in control solutions of urea in water. It is apparent that the lower results follow no regular sequence and vary considerably in the same solutions.

Table II illustrates the effect of sodium fluoride and thymol when added in varying amounts to a solution of urea in water and on the urea value in two cases of oxalated blood having a concentration of urea considerably above normal. These analyses bring out clearly the fact that sodium fluoride in greater concentrations than 10 mg. for each cc. lowers the urea value to a greater extent than the procedure recommended by Sander. This is not so apparent, however, when the urea is present in blood instead of in a pure water solution.

Table III gives a series of analyses of bloods to which varying

TABLE IV.

Effect on Urea Value in Water and in Blood of Increasing Incubation Period in Absence and Presence of Sodium Fluoride and Thymol Preservative.

Time of incubation, *min.*	30	90	150	210	270	330	390	450
	Mg. urea per 100 cc.							
Urea in water	124							
" " " + sodium fluoride and thymol.	30	118	112	116	118	116	118	116
Oxalated blood.	162	166	164	160	162			
" " + sodium fluoride and thymol.	106	166	158	160	160			

amounts of sodium fluoride and thymol mixture had been added. The preservative in a concentration of 3 mg. for each cc. has no significant effect on the urea value. The preservative in a concentration of 10 mg. or more for each cc. does lower the urea value. This is most striking in bloods with a high urea concentration and in bloods to which urea has been added.

These analyses have tended to show that the action of sodium fluoride and thymol is to retard the action of the enzyme. It seemed likely that if the enzyme were allowed a longer incubation period it would ultimately convert all the urea into ammonium carbonate. This fact is brought out in Table IV in which it is shown that if the enzyme in water solution and in blood is allowed only the routine time of 30 minutes to act on urea in the presence of sodium fluoride and thymol the urea value is very low, but that, if this

time of incubation is increased from 30 to 90 minutes or longer, the urea values approximate the quantity of urea as determined in the presence of potassium oxalate.

SUMMARY.

Sodium fluoride and thymol in the proportion of 10:1 and in the concentration of 10 or more mg. for each cc. of blood as recommended by Sander (7) lead to erroneous urea values as determined by the Van Slyke and Cullen modification of the Marshall urease method. The action of the preservative is to retard the conversion of urea to ammonium carbonate by the enzyme. Urea when added to either water or blood cannot be recovered quantitatively by the Van Slyke and Cullen procedure in the presence of sodium fluoride. If a longer period of incubation is used the urea values approximate more closely their true value. This procedure, however, is not recommended.

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THE FATE OF SUGAR IN THE ANIMAL BODY.

VIII. THE INFLUENCE OF INSULIN ON THE UTILIZATION OF GLUCOSE, FRUCTOSE, AND DIHYDROXYACETONE.

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I.

INTRODUCTION.

A balance for ingested glucose, with and without the administration of insulin, has been reported in a preceding paper (1). These experiments were made on rats fasted previously for 48 hours. A series of recovery experiments with fructose was then undertaken, on both 48 and 24 hour fasting rats (2). The use of animals fasted the shorter period was made possible by the fact that their preformed glycogen showed only a small individual variability, which is a necessary supposition in a determination of glycogen formation. The rats fasted a shorter period absorbed decidedly more fructose than those fasted a longer time. Subsequently, the same result was found to be true for glucose. This made it desirable to repeat the recovery experiments with glucose on 24 hour fasting rats. There was the possibility that the shorter fasting period had not only an influence on the rate of absorption, but

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also on the metabolic effect produced by insulin. This assumption proved to be correct, as is shown in the present paper.

Insulin decreases glycogen storage in the liver, after glucose feeding. In 48 hour fasting rats the surplus of sugar thus made available is disposed of, mainly, by increased oxidation; while in 24 hour fasting rats it is disposed of, mainly, by increased deposition of muscle glycogen.

Fructose differs from glucose in this respect. In previous experiments, insulin injections plus fructose feeding resulted in an increase in sugar oxidation, not only in 48 hour fasting, but also in 24 hour fasting rats. For the 48 hour series, glycogen determinations in the liver were available, which showed that insulin inhibits glycogen storage in this organ after fructose feeding. However, in the 24 hour series, glycogen formation in the liver and in the rest of the body had not been determined separately. An increased deposition of muscle glycogen under the influence of insulin might, therefore, have escaped attention. It was not expected that this increase would be very marked, on account of the strong increase in sugar oxidation, which would leave a smaller surplus of sugar for storage in the muscles. Nevertheless, this point seemed to merit further investigation in view of the fact that increased deposition of muscle glycogen is such a prominent feature after the administration of glucose plus insulin to 24 hour fasting rats.

Another reason for repeating the fructose experiments on 24 hour fasting rats, with separate determination of glycogen storage in the liver and the rest of the body, was that a fructose of higher purity was available for use at this time.

The fate of ingested dihydroxyacetone, with and without the injection of insulin, has also been investigated. This triose has received much attention recently. One group of investigators holds that dihydroxyacetone is converted into glucose by the liver. In this case, the fate of dihydroxyacetone should be the same as that of glucose. Another group of workers believes that this triose is a true intermediary of glucose catabolism and that it is utilized directly in both the normal and diabetic organism. The latter assumption presupposes that insulin has its point of attack before the triose stage is reached. In this case, insulin should be without influence on the fate of dihydroxyacetone in the animal body.

II.

Methods.

The aim of the present experiments was the same as in previous papers of this series; namely, to present a complete balance sheet for ingested sugar. For this purpose, the nitrogen excretion, sugar absorption and oxidation, and the glycogen formation in the liver and the rest of the body were determined on the same animal.

Male rats, reared in the laboratory on a standard diet,[1] were used. There was a surplus of food in the cages at all times. At noon, animals weighing 135 to 155 gm. were isolated and allowed to remain without food until the next day. The urine from the 4th to the 21st hour of fasting was collected quantitatively for nitrogen determinations. As a preservative, copper sulfate was added to the urine. After 21 hours of fasting, a metabolism fore period of 2 to 3 hours duration was made in a Haldane type of metabolism apparatus. Enough of the sugar under investigation was then fed by stomach tube to allow the absorption to proceed for 5 to 6 hours. There are two groups of experiments for each sugar. In one group the sugar was fed alone; in the second group insulin was injected immediately after the sugar feeding. The dose of insulin injected was 15 units per 100 gm. of body weight in the case of glucose, and 5 units in the case of fructose and dihydroxyacetone. The sugar feeding was followed immediately by a second metabolism period of 4 hours. The animals were then killed and worked up in the usual manner for the determination of sugar absorption and of glycogen content in liver and the rest of the body. For this purpose, the intestinal tract was removed as quickly as possible and the remainder of the carcass frozen with CO_2 delivered from a tank in order to reduce postmortem glycogenolysis. The liver was then removed and kept frozen until it could be analyzed separately for its glycogen content; in the meantime the rest of the body was cut into small pieces before being introduced into the boiling 60 per cent KOH for determination of its glycogen content.

[1] This diet consisted of whole wheat, 50 per cent; ground yellow corn, 20 per cent; skimmed milk powder, 10 per cent; ground fish and casein, 7 per cent each; and butter fat, 6 per cent.

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Control rats, which were treated in the same way with the exception of the sugar feeding, served for the determination of the preformed glycogen; that is, of the amount of glycogen present in the liver and the rest of the body at the time of the sugar feeding. There are two series of such determinations in the present paper, one coinciding with the glucose experiments (February to April, 1927), and a second, which was made at the time of the investigation on fructose and dihydroxyacetone (October to November,

TABLE I.

Glycogen Content of 24 Hour Fasting Rats.

Body weight.	Liver in per cent of body weight.	Glycogen content per 100 gm. of body weight.			Blood sugar.
		In liver.	In other tissues.	Total.	
Controls for glucose experiments.					
gm.	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*
166	3.56	7.4	124.6	132	97
149	3.80	8.1	141.9	150	98
140	3.41	8.2	127.8	136	92
157	3.43	6.6	142.4	149	96
153	3.71	7.6	124.4	132	101
	3.58	7.6	132.4	140	97
Controls for fructose and dihydroxyacetone experiments.					
123	3.42	3.0	123.0	126	85
132	3.58	4.2	129.8	134	93
130	3.62	9.9	144.1	154	87
141	3.48	7.1	114.9	122	81
	3.52	6.0	128.0	134	87

.1927). In order to find the amount of glycogen actually formed during 4 hours of sugar absorption, the preformed glycogen must be subtracted. This has been done in each case, the average values of Table I being used. The error introduced in this way is small since there is no marked individual variability in the preformed glycogen. Experience of the past 2 years has shown that there is a great constancy of the preformed glycogen from year to year, as well as at different seasons of the year. In two previous

series on 24 hour fasting rats, averages of 0.150 and 0.141 per cent total glycogen have been found, as compared with 0.140 and 0.134 for the averages in Table I. It is of considerable interest that the preformed glycogen of 48 hour fasting rats is only slightly lower. In two different series values of 0.114 and 0.136 per cent have been obtained. The significance of this fact and its connection with the problem of gluconeogenesis will be discussed in a later paper.

Calculations of the amount of sugar and fat oxidized have been made from the non-protein R.Q. by the use of the Zuntz-Schumburg-Lusk tables. In carrying out the metabolism determination the temperature of the animal chamber was, as usual, kept between 28–29°. Temperature regulation offered no difficulty, since the whole metabolism outfit, including the analytical balance, was set up in a room with automatic temperature regulation. In view of the insulin injections, special attention has been paid to muscular movements. Since the animal chamber is made of glass, the rats can be observed at all times. No difference was noted in the muscular activity of the rats receiving sugar alone and those receiving insulin in addition. This is ascribed to the fact that overdosage of insulin was avoided. During a metabolism period of 4 hours, muscular movements cannot be excluded altogether, but they are not a prominent feature with rats, since these animals are either asleep for most of the time or remain quiet when they are awake. As a rule, movements are confined to cleaning between periods of sleeping. The rats used were made tame and gentle by frequent handling. The absence of any fear reactions to ordinary procedures and the low degree of excitability make such animals especially suitable for work on carbohydrate metabolism. The sugar feeding, as practiced in this laboratory, does not cause any restlessness of the rats.

Previous metabolism work, including the glucose experiments of the present paper, has been made in a closed type of metabolism apparatus, which combines the Haldane principle with a direct O_2 determination by means of a spirometer. This apparatus was constructed 2 years ago, in order to check the reliability of the Haldane method, and has been in continuous operation since that time. In over 200 metabolism experiments, the agreement between the indirect O_2 determination according to Haldane's

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method and the direct O_2 determination was very satisfactory. Figures to this effect have been published in previous papers. The average difference between the two determinations of the present series was 1.2 ± 0.7 per cent. These extensive control experiments have convinced us that the Haldane method is very reliable and accurate if it is properly executed and if the metabolism periods are of sufficient length. Therefore we felt that there was no need to continue work with the closed system which is very time-consuming and rather cumbersome to handle. For this reason, the experiments with fructose and dihydroxyacetone have been made with the Haldane method plus the modifications described in our original paper (1). As an additional precaution a second soda-lime tower and sulfuric acid bottle have been added.

The dihydroxyacetone, as obtained from the Farbwerke vorm. Meister Lucius und Brüning, Hoechst-am-Main, after drying over P_2O_5, had a melting point of 75–76°. This agrees well with figures given in the literature (3). The substance was recrystallized from absolute alcohol and ether. To 10 gm. of the triose 30 cc. of absolute alcohol were added and, after cooling to 2°, 90 cc. of dry ether were added in small portions. The recrystallized product had a melting point of 80–81°, showing that probably a small amount of the polymeric form of the triose had been formed, which has a melting point of 155°. The product before and after recrystallization gave the same amount of reduction with Campbell's molybdenum method (4). 1 cc. of 0.01 N $KMnO_4$ was equivalent to 0.175 mg. of the triose in both cases. However, with the Hagedorn and Jensen method and the Bertrand method the reduction increased after recrystallization. The reason for this change in reducing power has not been ascertained. All metabolism experiments were made with the non-recrystallized triose.

Dihydroxyacetone reduces the ferricyanide solution of Hagedorn and Jensen and the alkaline copper sulfate solution of Bertrand in the cold. The reduction is not complete in either case, even on prolonged standing. In the case of the Bertrand solution the reduction in the cold reaches 92 per cent on 5 minutes standing as compared with the reduction after 3 minutes boiling. Standing up to 60 minutes in the cold did not increase the reduction. It was hoped that reduction in the cold might be used as a basis for quantitative determination since this would allow separation from

glucose and other sugars. It was found, however, that filtrates from biological material contain substances which inhibit reduction in the cold. Due to varying degrees of inhibition, the results are very erratic. This was the case especially in filtrates from intestinal washings. For this reason reduction in the cold could not be used.

It was necessary to establish the copper equivalents of dihydroxyacetone, since this sugar is not included in the Bertrand tables. To 20 cc. of the sugar solution, prepared from the unrecrystallized product, 20 cc. each of the two Bertrand solutions were added. The different samples were brought to the boiling point in the same length of time and the boiling was continued for exactly 3 minutes. The permanganate solution used for the

TABLE II.

Copper Equivalents of Dihydroxyacetone (Bertrand Method).

Sugar.	Cu	Cu equivalent per mg. of sugar.
mg.	mg.	
7	8.7	1.24
10	12.4	1.24
12	15.3	1.27
15	19.3	1.29
20	25.7	1.29
25	31.4	1.27

titration was standardized against C.P. glucose. Table II shows that between 7 and 25 mg., the reduction is proportional to the amount of triose present. The reduction beyond 25 mg. has not been investigated, but below 7 mg. the proportionality was less satisfactory.

Due to the great lability of dihydroxyacetone as compared with other sugars, the following control experiments have been performed in conjunction with the determination of absorption. Known amounts of the triose were added to the intestinal washings prior to the precipitation with colloidal iron. No loss occurred, which shows that the triose is not precipitated by the colloidal iron. The triose was added before extraction of the intestines with repeated portions of boiling water. This did not cause any loss of sugar.

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In urine the triose was determined by both the Bertrand and the Campbell procedures. The agreement between the two methods was very good. This indicates that there was no glucose or other sugar excreted besides the triose, since glucose gives with the Campbell reagent only $\frac{1}{180}$ of the reduction of dihydroxyacetone, while glucose reduces the Bertrand solution nearly twice as much as an equivalent amount of triose. Normal rat urine gives a strong blank with the Campbell reagent. Due to the large amount of triose excreted by the rats, the urine was diluted approximately 100 times, which obviated the use of a correction for the urine blank.

In blood the total sugar content was estimated by the Hagedorn and Jensen method (5), while the dihydroxyacetone was determined by means of the Campbell titration method (4). With the former method 0.1 mg. of dihydroxyacetone corresponds to 0.134 mg. in terms of glucose. By subtracting the glucose equivalent of the triose from the total sugar content, the glucose content of the blood plus other reducing substances (the so called residual reduction) is found by difference. Normal rat blood, when analyzed by the Campbell method, was found to contain an average of 12 mg. of reducing substances in terms of dihydroxyacetone. This blank was subtracted in all cases.

Fructose prepared from inulin, a C.P. product of the Pfanstiehl Company, was used for the present work. This sugar is of a high degree of purity and is only slightly colored when made up to 50 per cent solution. In previous experiments a C.P. fructose prepared from invert sugar was used. A 50 per cent solution of this sugar was highly colored which necessitated removal of the coloring matter with charcoal. It appeared later that different lots of that fructose were not absorbed by the rats at the same rate. Whether this was due to varying degrees of impurities present or to other factors inherent in the sugar, was unfortunately not established at that time. The same difficulty was encountered with dihydroxyacetone. When the greater part of the experiments had been completed, a new sample had to be opened. The absorption was consistently lower with the new sample. The experiments (two each in the series with and without insulin) in which the new sample of dihydroxyacetone was employed, are indicated by a dagger in Table X.

III.

Experiments with Glucose.

The individual experiments, with and without insulin, arranged according to the amount of glucose oxidized, are recorded in Table III. The average values have been calculated in Table XI.

TABLE III.

Recovery Experiments with Glucose.

Average weight of rats of glucose series, 129.9 gm.; of glucose plus insulin series, 130.7 gm. Values calculated per 100 gm. of body weight per 4 hours.

Fore period.		Absorption period.										
				Glycogen formed.								
O_2	R.Q.	Glucose absorbed.	Sugar oxidized.	In liver.	In rest of body.	Total.	Glucose recovered.	O_2*	R.Q.	Urine N.	Blood sugar.
Without insulin.											
gm.		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*		*mg.*	*mg.*
0.975	0.721	1.013	0.381	0.166	0.281	0.447	0.828	81.8	0.811	0.868	15.48	149
1.004	0.713	1.126	0.383	0.258	0.291	0.549	0.932	82.8	0.916	0.855	20.91	163
0.980	0.702	1.017	0.407	0.221	0.227	0.448	0.855	84.1	0.888	0.863	15.38	173
0.921	0.735	0.881	0.418	0.189	0.201	0.390	0.808	91.7	0.874	0.869	15.02	156
0.922	0.702	0.992	0.444	0.164	0.250	0.414	0.858	86.5	0.870	0.880	15.48	185
0.892	0.703	1.228	0.454	0.202	0.327	0.529	0.983	80.0	0.922	0.872	13.49	151
		1.075	0.522	0.154	0.230	0.384	0.906	84.2	0.891	0.904	16.72	164
0.910	0.713	1.144	0.525	0.170	0.334	0.504	1.029	90.0	0.917	0.896	13.02	148
1.098	0.722	1.094	0.539	0.141	0.289	0.430	0.969	89.1	0.962	0.894	14.74	152
0.885	0.720	1.079	0.568	0.257	0.199	0.456	1.024	94.9	0.801	0.945	16.24	138
With insulin.												
0.880	0.704	0.993	0.464	0.053	0.355	0.408	0.872	87.8	0.850	0.890	14.50	101
0.935	0.709	1.141	0.500	0.066	0.455	0.521	1.021	89.5	0.886	0.896	14.55	79
0.842	0.707	1.058	0.510	0.084	0.373	0.457	0.967	91.4	0.908	0.894	14.45	65
0.999	0.704	1.159	0.523	0.075	0.423	0.498	1.021	88.1	0.932	0.897	17.96	76
1.118	0.726	1.264	0.559	0.117	0.412	0.529	1.088	86.2	1.005	0.892	14.29	69
0.898	0.706	1.170	0.590	0.079	0.406	0.485	1.075	91.9	1.012	0.901	16.12	50
1.109	0.728	1.112	0.705	0.048	0.320	0.368	1.073	96.5	0.944	0.953	16.07	65

* Average value of direct and indirect O_2 determinations. The average difference between the determinations of the whole series was 1.2 ± 0.7 per cent.

There is a close agreement in the average O_2 consumption and the R.Q. of the fore period of the two groups of rats. After a 24 hour fast, the R.Q. of the rat stands at the fat-protein level, while oxidation of preexisting carbohydrate reserves is absent. This agrees well with the observation, recorded in a previous section, that the total glycogen content of the rat undergoes only a slight diminution between the 24th and 48th hour of fasting.

On an average, the animals receiving insulin absorbed 5.9 per cent more glucose than the rats without injections. In 24 hour fasting rats insulin seems to accelerate to a slight extent the rate of intestinal absorption since the same observation has also been made with dihydroxyacetone. The average amount of absorbed glucose accounted for was 86.5 per cent for the control and 90.2 per cent for the insulinized rats. This agrees well with the recovery obtained on 48 hour fasting rats. If insulin would lead to the formation of some unknown intermediary substance, as Macleod is still inclined to believe, the recovery of absorbed glucose should be lower in the insulin experiments; this is, however, not the case. It will be investigated in the near future, whether or not the loss of 10 to 14 per cent of absorbed glucose is of any significance from the standpoint of metabolism, or is merely due to errors of methods used and a possible slight inaccuracy of the Zuntz-Schumburg formula. Recovery experiments extending over longer periods should give a satisfactory answer to this question.

The total amount of glycogen formed is practically the same in the two groups of rats, while glucose oxidation is 18.3 per cent higher in the insulinized group. However, these two factors do not fully reveal the changes in the disposal of sugar for which insulin is responsible. In order to appreciate the true nature of insulin action, glycogen formation in the liver and the rest of the body has to be compared in the two groups of rats. This is shown in Table XI. It will be noted that insulin suppresses glycogen formation in the liver, while it increases glycogen deposition in the rest of the body.

It is of interest to compare the utilization of glucose in 24 and 48 hour fasting rats. The disposal of ingested glucose in 24 hour fasting (non-insulinized) rats differs in two respects from that of 48 hour fasting rats. In the first place, the rate of intestinal absorption is much greater in the former group; namely, 1.065 gm.

of glucose per 100 gm. of body weight per 4 hours, as against 0.750 gm. for the animals fasted the longer period. On account of the larger amount of glucose absorbed, both oxidation and glycogen formation are higher in animals fasted previously for a shorter period. The average values per 100 gm. of body weight per 4 hours are: 465 mg. oxidized and 455 mg. deposited as glycogen for the 24 hour fasting rats (Table XI), and 281 mg. oxidized and 388 mg. converted into glycogen for the 48 hour fasting rats (1). Secondly, for an equal percentage of absorbed glucose accounted for by these two processes, the quotient $\frac{\text{glycogen formed}}{\text{glucose oxidized}}$ is 1.00 for the former and 1.38 for the latter animals. It means that the 24 hour fasting rats oxidize relatively more glucose and deposit relatively less glycogen. This becomes evident if one

TABLE IV.

Sugar Oxidation and Glycogen Formation Calculated in Parts per 100 Parts of Absorbed Glucose.

	Oxidized.	Deposited as liver glycogen.	Deposited as body glycogen.
48 hr. fasting	38	16	36
24 " "	44	18	25
Difference	+6	+2	−11

calculates the respective values per 100 parts of absorbed glucose, as is shown in Table IV. The chief difference is in the relative rate of sugar oxidation and glycogen deposition in the muscles, while there is no marked change in the relative amount of glycogen formed in the liver. It is of interest that longer fasting has a depressing effect on sugar oxidation and that this is counterbalanced by increased glycogen deposition in the muscles. Probably for this reason insulin stimulates chiefly oxidation in 48 hour fasting rats.

The response to insulin of the 24 hour fasting rat differs only quantitatively from that of the 48 hour fasting rat. Due to the greater rate of absorption, the absolute values for glucose oxidation and glycogen formation are higher in the former animals; namely, 550 mg. oxidized and 467 mg. deposited as glycogen (Table XI) against 378 mg. oxidized and 324 mg. converted into

glycogen for the 48 hour fasting rats (1). However, the relative amounts of glucose oxidized and glycogen formed, remain the same in both cases, as is evidenced by the quotients $\frac{\text{glycogen formed}}{\text{glucose oxidized}}$ of 0.86 and 0.85 respectively (see Table V). It will be noted that the difference in the disposal of glucose between 24 and 48 hour fasting rats disappears when insulin is injected. How this is brought about is shown in Table VI by a rearrangement of Tables IV and V.

TABLE V.

Sugar Oxidation and Glycogen Formation Calculated in Parts per 100 Parts of Absorbed Glucose.

	Oxidized.	Deposited as liver glycogen.	Deposited as body glycogen.
48 hr. fasting plus insulin	50	4	38
24 " " " "	49	6	36
Difference	−1	+2	−2

TABLE VI.

Sugar Oxidation and Glycogen Formation Calculated in Parts per 100 Parts of Absorbed Glucose.

	Oxidized.	Deposited as liver glycogen.	Deposited as body glycogen.
48 hr. fasting without insulin	38	16	36
48 " " with insulin	50	4	38
Difference	+12	−12	+2
24 hr. fasting without insulin	44	18	25
24 " " with insulin	49	6	36
Difference	+5	−12	+11

In 48 hour fasting rats, insulin increases sugar oxidation to the same extent as it decreases glycogen formation in the liver. That is to say, the 12 parts of absorbed glucose which are oxidized in excess correspond to the 12 parts which fail to be deposited as liver glycogen. There is no need to assume that the lessened deposition of liver glycogen is due to the release of epinephrine, since the same effect is observed on adrenalectomized rats receiving glucose plus insulin (6). On the other hand, there is no marked change in glycogen deposition in the muscles, though there is evidence that the increased sugar oxidation occurs chiefly in them.

As has been pointed out in a previous paper, this has been difficult to reconcile with the view of Bissinger and Lesser (7) that sugar oxidation and glycogen formation in the muscle is a coupled reaction and that it is accelerated by insulin. However, in 24 hour fasting rats, an increased deposition of muscle glycogen takes place following insulin injections. Here the 12 parts of absorbed glucose, that fail to be deposited in the liver of the insulinized rats, are not entirely accounted for by increased oxidation, as is the case in 48 hour fasting rats. It will be noted that only 5 parts of the absorbed glucose are oxidized in excess, the remaining parts being accounted for by increased glycogen deposition in the rest of the body (chiefly muscles).

A surplus of insulin is therefore seen to promote both oxidation and storage of sugar in glucose-fed rats and it depends on the relative rates of these two processes in the non-insulinized animal as to which of the two will be influenced most when insulin is injected. In 48 hour fasting rats, in which glucose oxidation is relatively low and glycogen formation in the muscles relatively high, insulin is seen to influence mainly the former process. In 24 hour fasting rats, where the conditions are reversed, that is where glucose oxidation is perhaps close to its maximum rate, while glycogen deposition in the muscles is relatively low, insulin accelerates mainly the latter process. Insulin may also promote glycogen storage in the liver, but this cannot be readily demonstrated experimentally in the normal animal because the injection of even a small dose creates an excess of insulin in the body; and this leads to a shift in the disposal of sugar from the liver to the muscles, with the result that less sugar is available for glycogen deposition in the liver. In order to demonstrate glycogen synthesis in the liver under the influence of insulin, one has to choose different experimental conditions. Thus it has been shown by one of the authors that insulin injections lead to an accumulation of liver glycogen in fasting depancreatized cats and dogs (8).

IV.

Experiments with Fructose.

The present experiments with this sugar (Table VII) confirm the results obtained in a previous paper (2), though the absolute values differ to a slight extent because absorption was 10.3 per

cent higher in the former series. In contrast to glucose and dihydroxyacetone, insulin causes a marked increase in fructose oxidation in 24 hour fasting rats. The average values recorded in Table XI show a difference in fructose oxidation between the control and the insulinized group of 63.2 per cent, while the difference for glucose and dihydroxyacetone is only 18.3 and 8.7 per cent respectively. In previous experiments the difference in fructose

TABLE VII.

Recovery Experiments with Fructose.

Average weight of rats of fructose series, 132.4 gm.; of fructose plus insulin series, 135.2 gm. Values calculated per 100 gm. of body weight per 4 hours.

Fore period.		Absorption period.										
				Glycogen formed.								
O_2	R.Q.	Fructose absorbed.	Sugar oxidized.	In liver.	In rest of body.	Total.	Fructose recovered.	O_2	R.Q.	Urine N.	Blood sugar.
Without insulin.											
gm.		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*		*mg.*	*mg.*
0.901	0.714	0.557	0.144	0.221	0.096	0.317	0.461	91.2	0.891	0.768	12.64	
1.035	0.715	0.509	0.171	0.219	0.056	0.275	0.446	83.9	0.936	0.771	11.23	143
0.897	0.709	0.490	0.225	0.167	0.055	0.222	0.447	87.5	0.804	0.805	11.13	135
1.014	0.716	0.611	0.243	0.219	0.051	0.270	0.513	82.9	0.882	0.804	12.44	137
With insulin.												
0.836	0.712	0.483	0.293	0.014	0.130	0.144	0.437	90.6	0.927	0.815	11.56	66
0.946	0.707	0.544	0.296	0.038	0.168	0.206	0.502	92.2	0.900	0.819	10.94	78
0.936	0.709	0.502	0.298	0.044	0.127	0.171	0.469	93.3	0.964	0.815	14.31	67
1.015	0.725	0.625	0.395	0.045	0.152	0.197	0.592	94.6	1.051	0.832	10.29	76

oxidation was 79 per cent in favor of the insulinized rats. However, glycogen formation in the liver and in the body had not been determined separately. An influence of insulin on glycogen storage in the muscles could, therefore, not be detected. The present experiments reveal that there is also an increased deposition of muscle glycogen when fructose is fed to insulinized rats. Table XI indicates how profoundly insulin affects the distribution of fructose between liver and muscles.

The recovery of absorbed fructose is 86.1 per cent for the control and 92.6 per cent for the insulinized group. In former experiments on 24 hour fasting rats the recovery was 81.2 and 89.7 per cent respectively. However, in 48 hour fasting rats, without the injection of insulin, fructose oxidation was very low and the recovery was only 59.2 per cent. That this was due to a lag in fructose oxidation, could be demonstrated in the following way. When the metabolism period was extended for 2 hours beyond the period of absorption, an additional amount of sugar was oxidized, resulting in a recovery of 95 per cent, while no extra amount of glycogen was deposited. The lag in fructose oxidation in 48 hour fasting rats disappeared when insulin was injected. This resulted in a

TABLE VIII.

Sugar Oxidation and Glycogen Formation Calculated in Parts per 100 Parts of Absorbed Fructose.

	Oxidized.	Deposited as liver glycogen.	Deposited as body glycogen.
48 hr. fasting without insulin	36 (10)	39	10
48 " " with insulin	57	5	26
Difference	+21	−34	+16
24 hr. fasting without insulin	36	38	12
24 " " with insulin	59	6	27
Difference	+23	−32	+15

marked increase in fructose oxidation and in a recovery of 87.8 per cent. After glucose feeding there is no lag in sugar oxidation in 48 hour fasting rats.

Another difference between these two sugars was detected when the intravenous tolerance of rats was investigated (9). With glucose there was no difference in tolerance between 48 hour fasting and non-fasting rats, and insulin injections increased the glucose tolerance by 20 per cent. With fructose the tolerance of 48 hour fasting rats was only one-half that of non-fasting rats. Insulin injections did not increase the tolerance for fructose, an observation which has also been made by Wierzuchowski (10) on dogs. Basch and Pollak (11) found that, with insulin, larger amounts of fructose could be injected intravenously without causing glycosuria, than without insulin. Since the whole amount of

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fructose was injected within a few minutes, the experimental procedure is not comparable to an intravenous infusion at a constant rate. This may account for the different results obtained.

As in the case of glucose, longer fasting reduces the rate of absorption of fructose. Since 48 and 24 hour fasting rats do not absorb the same amount of fructose, a comparison must be made on the basis of 100 parts of absorbed sugar (see Table VIII). The values for the 48 hour fasting rats have been calculated from a previous paper (2). Since there is a lag in fructose oxidation in 48 hour fasting rats, two values are recorded. The value in parentheses corresponds to what is actually observed in a 4 hour absorption period; the second value has been calculated from the experiments in which the lag was compensated by extending the metabolism period beyond the period of absorption. It will be noted that the disposal of absorbed fructose is the same in 48 and 24 hour fasting rats, in spite of the fact that the rate of absorption is different in the two cases. Furthermore, when insulin is injected, the same type of changes are produced in the two groups of rats. There is an increase in glycogen deposition in the muscles and a still greater increase in sugar oxidation. This leaves almost no sugar for glycogen deposition in the liver, so that 33 parts of absorbed fructose fail to appear as liver glycogen.

The importance of the liver for the disposal of fructose as compared with other sugars, is shown by the following figures. Per 100 parts of absorbed sugar, 38 parts of liver glycogen are formed from fructose, but only 18 parts from glucose, and 21 parts from dihydroxyacetone.

In a determination of the intravenous tolerance of fructose, the low kidney threshold for this sugar must be taken into consideration. This is illustrated by the following experiments. In non-fasting rats the tolerance for fructose is 4 times lower than for glucose. However, when glucose or fructose is infused markedly above the tolerance rate (4.5 gm. per kilo in each case), a procedure which makes the difference in the kidney threshold less significant, there is no marked difference in the rate of utilization of these two sugars (9).

The reduced intravenous fructose tolerance of 48 hour fasting rats as compared with non-fasting rats is ascribed to the lag in

sugar oxidation which occurs in the former animals. Reinhold and Karr (12) found recently that a 4 day fasting period reduces the fructose tolerance of rabbits very markedly. There is no indication that glycogen formation in the liver or in the muscles is affected by longer fasting. During fructose absorption liver and body glycogen were formed at the same relative rate in 48 and 24 hour fasting rats, as Table VIII has shown. During intravenous infusion into a peripheral vein, the fructose passes directly into the general circulation and is carried past the kidneys before it reaches the liver. This is an additional factor which tends to lower the tolerance, because the liver is of special importance for the removal of fructose from the blood stream. That this is actually so has been demonstrated in the following way. When intestinal absorption was imitated by infusing fructose into a mesenteric vein, the intravenous tolerance of 48 hour fasting rats was doubled. The fructose tolerance of non-fasting rats (for an infusion into a peripheral vein) is higher than that of 48 hour fasting rats, because no lag occurs in sugar oxidation. The factors discussed above do not apply to glucose, because the kidney threshold for this sugar is high and because glucose oxidation in the rat is not reduced by fasting.

Since insulin accelerates the disposal of fructose in the peripheral tissues, one should expect an increase in intravenous fructose tolerance when insulin is injected. This is, however, not the case. The tolerance is a measure of the maximum capacity of the tissues to withdraw sugar. In the case of fructose, the withdrawal has to be very rapid, in order to avoid glycosuria, because the kidney threshold for this sugar is very low. During fructose absorption, insulin accelerates both sugar oxidation and glycogen formation in the muscles, but this does not lead to the disappearance of more sugar, because the same amount of sugar is absorbed by the non-insulinized and insulinized animals; it merely results in a corresponding decrease in glycogen formation in the liver. This shift of sugar from the liver to the peripheral tissues involves 33 per cent of the absorbed sugar in the case of fructose, but only 16 per cent in the case of glucose. Consequently, the peripheral tissues have a smaller range for the disposal of an additional amount of fructose than of glucose. This might be one of the reasons why insulin increases the intravenous tolerance for glucose but not for

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fructose. Insulin does not reduce the dihydroxyacetone content of the blood of rats. It is not known whether the same is true for fructose.

TABLE IX.

Amount of Dihydroxyacetone Excreted during 4 Hours of Absorption.

The urine values have been calculated per 100 gm. of body weight per 4 hours.

Body weight.	Total blood sugar.	Triose in blood.	True blood sugar by difference.*	Triose in urine.
		Without insulin.		
gm.	*mg.*	*mg.*	*mg.*	*mg.*
130	112	26.0	93	15.5
134	157	9.8	150	
135				44.3
162	106	4.0	103	27.0
161	116	8.8	109	23.3
162	111	9.3	104	28.9
	120	11.6	112	27.8
		With insulin.		
130	109	46.5	74	40.8
136	162	16.9	149	57.0
140	152	70.2	100	54.9
127	130	7.6	124	36.3
161	58	6.2	53	39.9
	122	29.5	100	45.8

* Total blood sugar minus dihydroxyacetone in terms of glucose.

V.

Experiments with Dihydroxyacetone.

1. Dihydroxyacetone Content of Blood and Urine.—The rate of absorption of the triose is intermediate between that of glucose and that of fructose. In contrast to glucose and fructose, glycosuria occurs when the triose is fed to rats. The sugar appearing in the urine is dihydroxyacetone. No evidence has been obtained that glucose is excreted in addition to the triose (see under "Methods").

The amount excreted per 100 gm. of body weight per 4 hours has been determined in a separate series of experiments (Table

TABLE X.

Recovery Experiments with Dihydroxyacetone.

Average weight of rats of triose series

TABLE X.

Recovery Experiments with Dihydroxyacetone.

Average weight of rats of triose series, 135.6 gm.; of triose plus insulin series, 136.6 gm. Values calculated per 100 gm. of body weight per 4 hours.

Fore period.		Absorption period.											
				Glycogen formed.									
O_2	R.Q.	Triose absorbed.	Sugar oxidized.	In liver.	In rest of body.	Total.	Triose recovered.	O_2	R.Q.	Urine N.	True blood sugar.*	Triose in blood.
Without insulin.												
gm.		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*		*mg.*	*mg.*	*mg.*
0.920	0.726	0.584†	0.315	0.128	0.080	0.208	0.523	89.4	0.847	0.835	14.09	100	15
0.880	0.723	0.786	0.330	0.164	0.147	0.311	0.641	81.6	0.871	0.838	14.53	163	36
0.860	0.735	0.610†	0.355	0.135	0.069	0.204	0.559	91.4	0.792	0.859	11.97		
0.899	0.726	0.738	0.371	0.173	0.077	0.250	0.621	84.2	0.866	0.851	12.01		
0.920	0.719	0.739	0.385	0.129	0.115	0.244	0.629	85.1	0.889	0.855	14.39	144	2
0.864	0.712	0.704	0.388	0.129	0.112	0.241	0.629	89.3	0.817	0.870	15.43	154	6
With insulin.													
0.885	0.711	0.590†	0.331	0.025	0.195	0.220	0.551	93.3	0.882	0.838	15.57		
0.869	0.732	0.801	0.367	0.044	0.262	0.306	0.673	84.0	0.980	0.835	14.43	138	14
0.923	0.713	0.803	0.383	0.014	0.271	0.285	0.668	83.2	0.945	0.845	14.39	74	34
0.943	0.709	0.695†	0.385	0.027	0.246	0.273	0.658	94.7	0.902	0.854	15.88	96	37
0.844	0.724	0.723	0.405	−0.003	0.242	0.239	0.644	89.1	0.903	0.858	13.26		
0.919	0.721	0.817	0.457	0.005	0.243	0.248	0.705	86.3	0.930	0.874	13.99	99	65

* Total blood sugar minus dihydroxyacetone in terms of glucose.

† Other sample of dihydroxyacetone.

IX). Insulin injections did not abolish the excretion. On the contrary, the insulinized rats eliminated an average of 46 mg., while the control rats excreted only 28 mg. This difference in excretion is also reflected in the triose content of the blood which was, as an average, 29.5 mg. per cent for the insulinized rats and 11.6 mg. per cent for the control rats. It is peculiar that the triose content of blood shows such a wide fluctuation; namely, from 6.2 to 70.2 mg. in the insulinized rats, and from 4.0 to 26 mg. in the control rats. This would seem to make the average values of little significance. However, a similar difference has been observed in the metabolism rats, the average triose content of the blood being 37.3 mg. per cent for the insulinized and 14.9 mg. per cent for the control rats (Table X). The true blood sugar (*e.g.* total blood sugar minus dihydroxyacetone in terms of glucose) was 112 mg. per cent for the control and 100 mg. per cent for the insulinized rats (Table IX). The respective values for the metabolism rats were 140 and 102 mg. per cent. In spite of the large dose of insulin injected, which produced marked metabolic changes, an actual hypoglycemia was missing. The same dose of insulin produced marked hypoglycemia during fructose absorption. The true blood sugar (glucose plus residual reduction) is slightly increased during dihydroxyacetone absorption in the non-insulinized rats. There are several points in connection with the dihydroxyacetone content of the blood and the influence of insulin thereon, which need further elucidation, but this was beyond the scope of the present paper.

It was not possible to determine the amount of triose excreted by the metabolism rats, because complete urine samples could not be obtained in all cases. On account of the sugar excretion, diuresis was larger than during glucose or fructose absorption, and some of the rats voided during the metabolism period. It was observed that the rats licked up the urine and it was necessary to increase the air current in order to dry the chamber. Complete absorption of CO_2 and water was ascertained for these conditions. Only such metabolism experiments are here reported in which the animal chamber was dry at the end of the period. The urine contained in the bladder was added to the intestinal washings. Since the amount of sugar absorbed is represented by the difference between the amount fed and the amount recovered from the intestine, the average absorption values for dihydroxyacetone,

recorded in Table XI, are slightly too low. They can be corrected by means of the average values of Table IX, by the addition of 29.0 mg. to the value for the control and of 46 mg. to that of the insulinized rats. Obviously, it is irrelevant for the calculation of the recovery of the absorbed sugar whether or not the triose excretion in the urine is determined separately.

TABLE XI.

Averages Calculated from Tables III, VII, and X.

All values per 100 gm. of body weight per 4 hours.

	Glucose.	Glucose plus insulin.	Fructose.	Fructose plus insulin.	Dihydroxyacetone.	Dihydroxyacetone plus insulin.
Fore period.						
O_2, *gm.*	0.954	0.969	0.962	0.933	0.890	0.897
R.Q.	0.715	0.712	0.714	0.713	0.724	0.718
Total calories	3.09	3.15	3.13	3.10	2.89	2.90
Sugar absorption period.						
Sugar absorbed, *gm.*	1.065	1.128	0.542	0.538	0.694	0.738
" oxidized, *gm.*	0.465	0.550	0.196	0.320	0.357	0.388
Glycogen formed in liver, *gm.*	0.192	0.075	0.206	0.035	0.143	0.019
" " " rest of body, *gm.*	0.263	0.392	0.065	0.144	0.100	0.243
Total glycogen, *gm.*	0.455	0.467	0.271	0.179	0.243	0.262
Sugar recovered, *gm.*	0.919	1.017	0.467	0.499	0.600	0.650
" " *per cent*	86.5	90.2	86.1	92.6	86.7	88.3
" lost, *gm.*	0.146	0.111	0.075	0.039	0.094	0.088
Glycogen formed / Sugar oxidized	1.00	0.86	1.38	0.56	0.68	0.68
Blood sugar, *mg.*	158	72	138	72	140*	121*
O_2, *gm.*	0.885	0.934	0.878	0.960	0.847	0.924
R.Q.	0.884	0.903	0.787	0.821	0.851	0.850
Urine N, *mg.*	15.65	15.42	11.86	11.77	13.74	14.58
Non-protein O_2, *gm.*	0.752	0.803	0.777	0.861	0.730	0.799
" R.Q.	0.900	0.921	0.786	0.823	0.860	0.859
Protein oxidized, *gm.*	0.096	0.095	0.073	0.072	0.084	0.089
Fat oxidized, *gm.*	0.090	0.076	0.199	0.181	0.122	0.134
Calories from protein†	0.39	0.38	0.30	0.29	0.34	0.36
" " fat‡	0.85	0.71	1.87	1.70	1.15	1.27
" " sugar§	1.74	2.06	0.73	1.20	1.34	1.45
Total calories	2.98	3.15	2.90	3.19	2.83	3.08

* Total blood sugar.

† Heat from protein (urine N × 24.98 calories).

‡ Heat value for animal fat 9.4 calories.

§ Heat value for sugar 3.74 calories.

2. Metabolism Experiments.—The individual experiments with dihydroxyacetone, recorded in Table X, need no special discussion. Four experiments (two without and two with insulin) marked with a dagger in Table X were made with a different sample of dihydroxyacetone. This has already been referred to under "Methods." The average values in Table XI show a close agreement in O_2 consumption and R.Q. of the fore period, which indicates that animals of a uniform character have been used for the two groups of experiments. On an average, the insulinized rats absorbed 6.3 per cent more dihydroxyacetone than the control rats. The recovery of the absorbed triose was 88.3 per cent for the insulinized and 86.7 per cent for the control group. This agrees well with the values obtained for glucose and fructose.

The average non-protein R.Q. of the sugar absorption period is the same in the two groups of rats. In comparison to the fore period the O_2 consumption shows a slight fall in the non-insulinized group and a slight rise in the insulinized group, the difference in sugar oxidation amounting to 8.7 per cent in favor of the latter group. A determination of the respiratory metabolism alone might have led to the erroneous conclusion that insulin has no effect on the disposal of dihydroxyacetone. The same fallacy would have been met with if only the total glycogen formation of the rats had been determined, which is within 7 per cent the same in the two groups. As in the case of glucose and fructose, the effect of insulin lies in the distribution of absorbed sugar between the liver and the rest of the body. Under conditions of intestinal absorption, insulin cannot lead to the disappearance of larger amounts of sugar, since the amount of sugar available to the two groups is practically the same. The shift in the disposal of dihydroxyacetone from the liver into the muscles, as the result of insulin injections, is shown in Table XII, where the respective values have been calculated per 100 parts of absorbed sugar.

VI.

Comparison of Glucose, Fructose, and Dihydroxyacetone.

The ratio in the rate of absorption of these three sugars in 24 hour fasting rats, is of the order 100:51:65. In 48 hour fasting rats, the ratio between glucose and fructose is of the order 100:43.

Since each sugar is absorbed at a different rate, a factor which has either been disregarded or minimized in previous experiments on men and animals, a comparison must be made for an equal amount of absorbed sugar. For absolute values, Table XI has to be consulted. It would, indeed, be misleading to compare the total amount of glucose and dihydroxyacetone oxidized in 4 hours, which is 0.465 and 0.357 gm., and to conclude from this that glucose is more easily oxidized in the animal body than the triose. Such a comparison gains an entirely different meaning, if it is based on 100 parts of absorbed sugar. The same applies to glycogen formation in the liver and the rest of the body.

In Table XIII, all values are based on 100 parts of absorbed sugar and are, therefore, expressed in per cent.

Each sugar has its own characteristic utilization in the animal body. This utilization is determined by the percentage of

TABLE XII.

Sugar Oxidation and Glycogen Formation Calculated in Parts per 100 Parts of Absorbed Dihydroxyacetone.

	Oxidized.	Deposited as liver glycogen.	Deposited as body glycogen.
Without insulin	51	21	15
With insulin	53	2	33
Difference	+2	−19	+18

absorbed sugar which is oxidized, deposited as liver glycogen, and deposited as body glycogen. These three processes are interdependent. An increase in one process necessarily means a decrease in one or two of the others. It is important to know which of the three processes is the determining factor in each case. With dihydroxyacetone, the larger percentage of sugar oxidation may leave less sugar for glycogen deposition in the muscles, but the opposite may also be true. With fructose, either increased glycogen formation in the liver or decreased glycogen formation in the body may be the determining factor for the characteristic utilization. As long as this is not definitely known, it is perhaps best to refrain from saying that one sugar is more easily oxidized in the body than another or that one sugar is a better glycogen former than another. The statement often occurs in the litera-

body weight per 4 hours, which is a good agreement, since the maximum difference is only 1.9 per cent. In former experiments on 24 hour fasting rats the average calorie output of eleven animals was 3.01 (2). Groups 5 and 6 of the present series show somewhat lower calorie output, namely 2.89 and 2.90.

When sugar is fed to 24 hour fasting rats, the metabolism is shifted from a combustion of oxygen-poor fat to that of oxygen-rich carbohydrates and this shift is accompanied by a fall in O_2 consumption and a slight decrease in heat production. After glucose feeding the heat production diminished 3.5, after fructose 7.3, and after dihydroxyacetone 0.2 per cent. In previous experiments with fructose the calories diminished 5 per cent (2). The lower heat production in the sugar absorption period as compared with the fore period may be due in part to the effect of caging. Presumably, a specific dynamic action of carbohydrates is not observed in rats fasted previously for 24 hours, because the animals start with an R.Q. of 0.71 and because the glycogen stores are low. Consequently, a condition of carbohydrate plethora (Lusk (15)) is not established until the glycogen stores have been replenished. This is supported by recent observations made in this laboratory, which show that a specific dynamic action is observed in 24 hour fasting rats when the glucose absorption has proceeded for 5 to 6 hours, at which time the R.Q. approaches unity. In the first 4 hours, equal parts of absorbed glucose are oxidized and stored as glycogen. In later periods of absorption, more sugar is oxidized than stored as glycogen and this is associated with a rise in O_2 consumption and heat production.

After sugar feeding plus insulin, the calories are equal to those in the fore period in the case of glucose and slightly higher than in the fore period in the case of fructose and dihydroxyacetone. The heat production in the experiments with sugar plus insulin is higher than in those with sugar alone. The increase amounts to 5.7 per cent with glucose, to 10.0 per cent with fructose, and to 8.8 per cent with dihydroxyacetone.

While Chaikoff and Macleod (16) found no reduction of O_2 consumption in insulinized rabbits and dogs, they regard the experiments of Dudley, Laidlaw, Trevan, and Boock (17) as evidence that O_2 consumption is reduced in mice after insulin. Dudley and collaborators injected convulsive doses of insulin into fasted mice

and noted an enormous fall in O_2 intake. In our experiments on rats, where hypoglycemic symptoms are prevented by sugar feeding, insulin does not have this effect. It will be shown in a later paper that the same is true for fasting rats, if they are rich in glycogen and if a non-convulsive dose is injected. On the other hand, the results of Dudley and collaborators were confirmed on fasting rats with poor carbohydrate reserves, when large doses of insulin were given (unpublished experiments). A decreased O_2 consumption and fall in body temperature has also been recorded in large laboratory animals after insulin convulsions. We find, therefore, no basis for the assumption of Chaikoff and Macleod that insulin has a different effect on the O_2 consumption of small laboratory animals as compared with large ones. In both types of animals the changes in O_2 intake are small if non-convulsive doses of insulin are injected.

Chaikoff and Macleod found that insulin has only a slight effect on the rate of carbohydrate oxidation of well fed rabbits. The same is observed in our present experiments on glucose-fed rats. However, their conclusion that "the sugar which insulin caused to disappear" may either have been deposited as glycogen or converted into some intermediary substance and the dismissal of the former possibility as unlikely, are open to criticism. The question is, what is meant by "the sugar which insulin caused to disappear." In order to be of significance, this must have a quantitative meaning. Obviously, the sugar available for conversion into glycogen or into an unknown substance will be represented by the difference between the amount of sugar absorbed and the amount of sugar oxidized. This presupposes that at least two factors must be determined in order to draw conclusions as to changes in the third. However, the amount of sugar absorbed or changes in glycogen have not been determined in these experiments. The animals were well fed for some days previously and food was also placed in the respiratory cabinet for the 6 hours that the experiment lasted. Under these conditions there is no means of telling how much sugar was available to the insulinized rabbits in comparison to the untreated ones. The experiments merely show that both groups of animals oxidized the same amount of sugar but it is impossible to know how much sugar was left over in the two groups, for other metabolic processes. Without

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this knowledge a discussion of the fate of disappearing sugar is without significance.

In order to understand the standpoint taken by Macleod and collaborators, it will be necessary to refer to another publication. Barbour, Chaikoff, Macleod, and Orr (18), investigating the influence of insulin on glycogen deposition in the liver and muscles of rats after carbohydrate feeding, have been able to confirm our previous results. These workers also found that insulin retards glycogen formation in the liver, and noted that large doses of insulin cause an increase in glycogen deposition in the muscles. Here again the amount of sugar absorbed from the intestine was not determined. Nevertheless, the statement occurs that "it is certain that glycogen formation cannot account for more than a part of the disappearing glucose," as if the amount of "disappearing glucose" were known. Proceeding from this statement these authors refer to metabolism experiments of their own, on rabbits, in which insulin caused an increase in carbohydrate oxidation of 0.1 gm. of glucose per kilo, and they conclude that "it seems clear that increased combustion of carbohydrates is not as a rule adequate in intact animals, to account for all that portion of the disappearing sugar which is not deposited as glycogen," as if it were permissible to apply observations on the rate of sugar oxidation in rabbits to rats. The last statement of Macleod and collaborators is contradicted by previous work of Bissinger and Lesser (7), Best, Dale, Hoet, and Marks (19), and Cori and Cori (1), who have not only been able to determine quantitatively how much sugar disappears but who have also succeeded in accounting for the disappearing sugar by determining glycogen formation and sugar oxidation on the same animal.

VIII.

DISCUSSION.

Lesser (20) found increased glycogen deposition in mice which were analyzed *in toto* after intraperitoneal injection of glucose plus insulin. The determination of the respiratory quotient revealed a greater rate of sugar oxidation. Dale and collaborators (19) have shown that intravenous infusion of glucose does not lead to glycogen deposition in the muscles of the eviscerated spinal prep-

aration of the cat. However, when insulin is injected, more glucose has to be infused in order to prevent the blood sugar from falling and there is a marked deposition of muscle glycogen. At the same time, sugar oxidation is also increased. Both Lesser and Dale conclude that insulin promotes sugar oxidation and storage in the muscles. In our previous experiments with glucose on 48 hour fasting rats, increased sugar oxidation was the most prominent feature after the insulin injection, and was accompanied by an almost complete suppression of glycogen formation in the liver. Under conditions of intestinal absorption, the amount of sugar available is the same in the normal and insulinized animals. In the latter, the increased sugar oxidation and decreased glycogen formation in the liver balanced each other. These experiments were repeated on 24 hour fasting rats because they absorb sugar at a much greater rate than the 48 hour fasted rats. Here again the insulinized animals have no more sugar available than the control animals and insulin is therefore unable to cause more sugar to "disappear." It can merely affect the mode of utilization of glucose in the body. The diminished glycogen store in the liver was equally marked, but, in contrast to 48 hour fasting rats, there was a considerable increase in muscle glycogen, while increased sugar oxidation was less prominent. Increased deposition of muscle glycogen and a suppression of glycogen formation in the liver have also been obtained with fructose and dihydroxyacetone when insulin was injected. In contrast to fructose and glucose, insulin hardly affected the rate of oxidation of dihydroxyacetone.

How far do these experiments, in which insulin is present in excess, help to explain the function of this hormone under normal conditions? The utilization of ingested sugar in the normal animal shows us what insulin does when it is released by the pancreas according to physiological needs of the body. With glucose 44 per cent of the absorbed sugar is oxidized, 18 per cent is deposited as liver glycogen, and 25 per cent is converted into body glycogen. Fructose and dihydroxyacetone show a different utilization in the normal organism; a larger percentage of the former sugar is converted into liver glycogen, while a larger percentage of the latter sugar is oxidized.

If insulin is lacking, owing to the removal of the pancreas,

ingested sugar can neither be stored as glycogen, nor can it be oxidized. The processes of sugar disposal may not be suppressed entirely in the diabetic organism, but they must occur at an infinitely slower rate than in the normal animal. Insulin would then act as an accelerator of these processes. The other extreme, represented by an excess of insulin, shows us which of the processes of sugar disposal are accelerated by the pancreatic hormone. As we have seen, the acceleration occurs mainly in the peripheral tissues and involves both glycogen formation and sugar oxidation. An acceleration of glycogen formation in the liver cannot occur under conditions of intestinal absorption, because the peripheral tissues, which form the largest mass of the body, appropriate most of the absorbed sugar.

The influence of insulin on glycogen formation in the liver is still an open question. Under normal conditions, both the absorbed sugar and the insulin given off by the pancreas pass through the liver before they reach the peripheral tissues. The liver has, therefore, the first choice. But insulin is also needed for the disposal of sugar in the peripheral tissues. The supply of insulin by the pancreas must be so adjusted that the sugar disposal in the peripheral tissues does not gain the preponderance over that in the liver. Indeed, the slight alimentary hyperglycemia is an indication that, under normal conditions, the peripheral tissues do not work to their full capacity. On the other hand, if the peripheral tissues gain the preponderance on account of an insulin injection, the liver is either forced to give up its glycogen or does not form glycogen at all. In the former case, one is dealing with an ephemeral glycogen formation; in the latter case, glycogen formation in the liver is inhibited. No decision has been reached as to the merit of either of these possibilities. The absence of glycogen in the diabetic liver does not necessarily mean that insulin is needed for the synthetic process in the normal liver, since secondary influences may account for the loss of liver glycogen after the removal of the pancreas. However, when insulin is injected into fasting, depancreatized dogs and cats, liver glycogen reappears in considerable amounts (8). In normal fasting animals, the majority of investigators observed a decrease in liver glycogen after insulin injections, but Frank, Hartmann, and Nothmann (21) claim

to have found an increase in liver glycogen in fasting rabbits after the injection of small doses of insulin.

The conception is not without merit that the mode of utilization of sugar in the normal organism is controlled (among other factors) by the opposing influence of the internal secretion of the pancreas and the adrenal medulla. It must, however, not be lost sight of, that the actual point of attack of insulin and epinephrine in the process of carbohydrate disposal remains still a mystery. The end effects produced by these hormones are known, but the actual mechanism involved has not been ascertained.

The rôle of absorption in metabolism experiments needs a special discussion. The intestines of the animals investigated in this respect (dog, Nagano (22); cat, Hewitt (23); rat, Cori (24)) are posessed of a high degree of selective permeability for sugars of a very similar configuration. To give an example: In the rat, glucose is absorbed 5 times as fast as its isomer mannose and twice as fast as the keto sugar fructose. The order in the rate of absorption of four hexoses and two pentoses was the same in the dog and in the rat, while in the cat, which has been tested for only three sugars, galactose held another position than in the dog and rat, being absorbed more slowly than glucose. In contrast to the intestine, various hexoses and pentoses are absorbed at the same rate from the peritoneal cavity (25). Selective permeability is, therefore, a characteristic of the epithelial lining of the intestine, a property which is also exhibited in the most striking manner by the tubular epithelium of the kidney (Hamburger (26)). It seems unlikely that selective permeability is an exclusive property of the intestine of the dog, cat, and rat; it is more probable that one is dealing here with a general biological law which applies to other species and to man as well. To be sure, one cannot apply quantitative observations made on one species to another. It would not be fitting to say that because the rat absorbs glucose twice as fast as fructose, the same must hold true for man. Indeed, there are indications that the opposite may be the case. The order in the rate of absorption of hexoses might vary in the different species. The argument that is put forward here is this: If selective permeability is a property of the intestine of all mammals, everything speaks against the assumption that different hexoses are absorbed at the same rate in man. Since it is not known at what rate each

particular sugar is absorbed from the human intestine,[2] an interpretation of metabolism experiments, in which different sugars are fed, is very difficult.

Another factor which we believe to be true, not only for the rat but also for other species, is absorption of sugar at a constant rate. Ingestion of 200 gm. of glucose instead of 100 gm. does not lead to an increase in the rate of absorption, as one might believe; it merely doubles the length of time until absorption is completed. Absorption is, therefore, comparable to an intravenous infusion of sugar at a constant rate, provided such an infusion is made into the portal circulation.

A third factor, which is of special significance, is the inhibition in absorption which occurs when two different sugars (28) or sugars and amino acids (29) are fed simultaneously. For instance, galactose is absorbed by 48 hour fasting rats at a rate of 1.96 gm. per kilo per hour. However, galactose is absorbed from a glucose-galactose mixture of equal parts at a rate of only 0.74 gm. per kilo per hour. A similar reduction occurs in the rate of absorption of glucose when galactose is being absorbed at the same time, the inhibition being mutual. The total amount of sugar absorbed from a glucose-galactose mixture is, therefore, not greater than if glucose or galactose are being absorbed alone.

Finally, the length of time of fasting and other bodily conditions such as adrenalectomy in the rat (6), have a profound influence on the rate of intestinal absorption. No doubt, as more attention is paid to intestinal absorption, several other factors beside those here enumerated will be discovered.

If these absorption factors are taken into consideration, many results, difficult of explanation, published in the literature, are amenable to a different and often more simple interpretation than the one given by the authors. This contention is illustrated by the following example. Folin and Berglund (30) found on the same subject that the excretion of galactose in the urine after the ingestion of 100 gm. of glucose plus 100 gm. of galactose is less than one-tenth as great as the excretion obtained from 100 gm.

[2] Wierzuchowski (27) found that glucose and fructose leave the stomach at the same rate. Since absorption of sugars from the stomach is insignificant, this bears no relation to the rate of absorption from the intestines.

of galactose when taken alone. A similar difference has been observed by Bodansky (31) on dogs, and by Corley[3] on rabbits. The same is true for rats (unpublished experiments), but here the simultaneous determination of absorption reveals that the basis for this phenomenon is the mutual inhibition in absorption which has been described above.

Benedict and Carpenter (32) confirmed and enlarged upon the original observation of Tögel, Brezina, and Durig (33), that in men, ingestion of fructose causes a more rapid and sustained rise in R.Q. than an equal quantity of glucose. This has also been found by a number of other investigators. It is of importance that Carpenter (34) failed to find the characteristic rise in R.Q. when fructose was administered by rectum. He explains this difference by the hypothesis that substances injected into the rectum are metabolized without the aid of hormones elaborated when the material is ingested by mouth. Differences in rate of absorption might afford a simpler explanation, since it is known that the absorbing capacity of the large intestine is inferior to that of the small intestine. The higher R.Q.'s after fructose than after glucose ingestion might be due to a greater rate of absorption of the former sugar or they may be the result of the structural difference between these two sugars. In order to decide between these two alternatives, it is proposed to infuse these sugars intravenously at the same rate into normal men and to determine the respiratory metabolism at the same time. This has the advantage that the amount of sugar supplied during the metabolism period is known. After the ingestion of sugar the time at which absorption comes to an end remains unknown and the metabolism cannot, therefore, be correlated with the period of active absorption.

In men, under basal conditions, sugar derived from the glycogen stores is being oxidized. Since it is not known to what extent fructose replaces the glycogen metabolism, the higher R.Q. curve after fructose ingestion might also be due to the fact that fructose is less effective in reducing glycogen utilization than an equal quantity of glucose. The same consideration applies to dihydroxyacetone.

In the experiments on rats all these difficulties are absent. The

[3] Corley, R. C., *J. Biol. Chem.*, 1928, lxxvi, 31.

24 hour fasting animal has an R.Q. of 0.71 at the time of the sugar feeding. Comparisons are based not merely on the R.Q. curve, but on the actual amount of sugar oxidized. By determining glycogen formation and sugar oxidation simultaneously, 90 per cent of the absorbed sugar is accounted for. This indicates that the Zuntz-Schumburg formula, which is based on the validity of the non-protein R.Q. as a true combustion quotient of a mixture of fat and carbohydrates, is not far off under the conditions of our experiments. The amount of sugar absorbed is also known in the rat experiments. Owing to the difference in rate of absorption of various sugars, a comparison of the absolute values for sugar oxidation and glycogen formation in the liver and the body has little significance. In order to see how various sugars are utilized in the body, a comparison has to be made for an equal quantity of absorbed sugar. Under these conditions, each of the three sugars was found to be utilized in a different way. Per 100 parts of absorbed sugar, a larger percentage of dihydroxyacetone was oxidized than of glucose and fructose. Glycogen formation in the liver was highest with fructose, and lowest with glucose, while the opposite was true for glycogen formation in the body. It is concluded that the configuration of the sugar molecule determines not only the rate of intestinal absorption but also the mode of utilization in the tissues.

The statement is often found in the literature that the blood sugar curve after the ingestion of sugar is an index of the rate of absorption. This is not correct, because the blood sugar level does not depend on absorption alone but on the ratio: $\frac{\text{rate of absorption}}{\text{rate of removal in tissues}}$. Calculations have been given in a previous paper (24), which show that even a slight disproportionality between these two rates has a marked effect on the sugar concentration in the blood. Two sugars which are absorbed at the same rate will give a different blood sugar curve, if the rate of removal in the tissues is different. Glucose and galactose are absorbed at nearly equal rates in the rat. The higher blood sugar curve during absorption of the latter sugar is due to its slower utilization by the tissues. On the other hand, two sugars or the same sugar, when absorbed at different rates, will give an identical blood sugar curve if the above ratio remains unchanged. Glucose

as well as fructose is absorbed much faster in 24 hour fasting than in 48 hour fasting rats, but the almost identical blood sugar curves do not reveal this difference in absorption.

Cathcart and Markowitz (35) found recently, in men, that glucose causes a leisurely rise in the R.Q., whereas other sugars (sucrose, galactose, levulose, and dihydroxyacetone) bring about a rapid rise in the R.Q. to unity. They state that "these differences cannot be attributed to differences in the rate of absorption as shown by blood sugar estimations." If the blood sugar curve is regarded as an index of absorption, it would seem logical also to regard the R.Q. curve as an index of absorption. A more rapid rise in the R.Q. might then signify a more rapid absorption. The conflicting evidence, thus obtained, reveals the impossibility of such conclusions. Most authors prefer to attribute differences in the R.Q. curve to a different utilization of the various sugars in the body. Those sugars which give a more rapid rise in R.Q. are said to be utilized better than those which give a slow rise. It has already been pointed out that the utilization of a sugar does not only depend on its oxidation but also on its conversion into liver and body glycogen and that these three processes of sugar disposal are interdependent. A larger oxidation necessarily means less glycogen formation with no possibility for deciding which of the two is the primary process.

The first investigation of the ate of dihydroxyacetone in the body was made by Mostowski (36), who found that feeding the triose to fasting chickens leads to an increase in liver and muscle glycogen. No other work on glycogen formation from dihydroxyacetone could be found in the literature, except a casual statement by Isaac and Adler (37) that the triose is a better glycogen former in the liver than glucose. In the rat 21 per cent of the absorbed triose and 18 per cent of the absorbed glucose are converted into liver glycogen. In the muscles of the rat glycogen formation from the triose amounts to 15 per cent and from glucose to 25 per cent.

Conversion of the triose into glucose has been observed by Embden, Schmidt, and Wittenberg (38) in perfusion experiments on livers of phlorhizinized dogs. Ringer and Frankel (39) were able to account for a large percentage of dihydroxyacetone fed to phlorhizinized dogs by the excretion of extra sugar (glucose). A similar observation has been made by Campbell and Markowitz

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(40) on depancreatized dogs. The conversion of the triose into glucose in the diabetic organism does not necessarily prove that such a conversion is a necessary step in the utilization of the triose in the normal organism.

In another paper the last named authors (41) kept depancreatized dogs on a diet containing glucose. Only small doses of insulin were injected, so that a considerable glycosuria persisted. Sugar excretion remained practically unchanged when dihydroxyacetone or fructose was substituted for glucose in the diet. The authors conclude that in the diabetic organism there is no preferential utilization of dihydroxyacetone and fructose over glucose, and they explain this by a conversion of the triose and of fructose into glucose prior to utilization in the tissues. Determinations of R. Q. (Mason (42), Lambie and Redhead (43)), of blood sugar (Isaac and Adler (37), Rabinowitch (44)), of blood phosphates (Lambie and Redhead (43), Rabinowitch (45)), in mild and severe diabetics have been interpreted in an opposite way; namely, that the triose is utilized directly in the tissues of the diabetic organism and that this sugar is disposed of without the aid of insulin. In diabetic patients the R. Q. showed a steeper rise after dihydroxyacetone than after glucose ingestion. The same has been shown by Joslin (46) for fructose. These experiments show that there exist differences in the utilization of the triose, of fructose, and of glucose in the human diabetic, but they offer no proof that these sugars are utilized better than glucose, since the greater rate of oxidation of the triose and of fructose may be counterbalanced by a decreased rate of glycogen formation.

In normal men (Mason (47), Lambie and Redhead (43), Cathcart and Markowitz (35)) and in dogs (Himwich, Rose, and Malev (48)) dihydroxyacetone also leads to a steeper rise in the R. Q. than glucose. In the rat 51 per cent of the absorbed triose and 44 per cent of the absorbed glucose are oxidized. The fact that dihydroxyacetone is an effective remedy against insulin convulsions is interpreted by Campbell and Hepburn (49) as proof that the triose is converted into glucose; while Kermack, Lambie, and Slater (50) assume that a direct utilization of the triose relieves the symptoms. In hepatectomized animals the evidence is also conflicting. Markowitz and Campbell (51) find no utilization of the triose, while Kermack, Lambie, and Slater

(50) believe that the triose is utilized. Loebel (52) and Meyerhof and Lohmann (53) found no indication that dihydroxyacetone is changed into lactic acid or oxidized by surviving tissues. This was true not only for brain and muscle tissue but also for the liver (53, 54). On the other hand, fructose and glucose were oxidized by these tissues. These experiments on isolated tissues offer no clue as to the mode of utilization of the triose in the intact organism.

The discussion of the literature shows that there is disagreement on two questions. The first question deals with the preferential utilization of the triose in the diabetic organism. The substitution experiments of Campbell and Markowitz (41) have offered definite proof that in the depancreatized dog dihydroxyacetone and fructose are not utilized better than glucose. The second questions deals with the mode of utilization of the triose in the normal organism; namely, whether the triose can be utilized directly or whether it has to be converted into glucose by the liver in order to be utilized. In normal men and in dogs the rate of oxidation of dihydroxyacetone differs from that of glucose. The same is true for the incomplete human diabetes. In the normal rat dihydroxyacetone is also utilized in a different way than glucose, being oxidized to a larger extent and forming less glycogen than glucose, which suggests, but does not prove, a direct utilization of the triose in the peripheral tissues.

Insulin has a marked effect on the utilization of the triose in the rat, since it leads to a strong increase in deposition of muscle glycogen. It has already been pointed out that the sugar made available for increased glycogen deposition in the muscles is paid for by a decreased glycogen formation in the liver. The question yet to be investigated is whether glycogen formation in the liver is inhibited by an excess of insulin or whether there is an ephemeral glycogen deposition in the liver. In the latter case the sugar made available for increased glycogen deposition in the muscles would be glucose and not dihydroxyacetone. The fact that insulin leads to an increase in the dihydroxyacetone content of the blood and to a larger excretion of the triose in the urine seems to speak against an ephemeral glycogen deposition in the liver. In this case the sugar made available for increased glycogen deposition in the muscles would be dihydroxyacetone. Definite proof to

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this effect would be of some importance, because it would indicate that insulin is still able to produce its characteristic effect when the triose stage of the sugar molecule has been reached.

IX.

SUMMARY.

1. The ratio in the rate of absorption of glucose, fructose, and dihydroxyacetone is of the order 100:51:65. 44 per cent of the glucose absorbed in 4 hours is oxidized, 18 per cent is converted into liver glycogen, and 25 per cent into body glycogen, corresponding to a recovery of absorbed sugar of 87 per cent. The values obtained for fructose are, 36 per cent oxidized, 38 per cent converted into liver glycogen, and 12 per cent into body glycogen. Of dihydroxyacetone 51 per cent is oxidized, 21 per cent deposited as liver glycogen, and 15 per cent as body glycogen. The configuration of the sugar molecule determines not only the rate of absorption but also the mode of utilization of the sugar in the body.

2. During sugar absorption, insulin injections cannot lead to the disappearance of more sugar, because the animals with and without insulin have the same quantity of sugar available on account of nearly equal absorption. After insulin injections 49 per cent of the glucose absorbed in 4 hours is oxidized, only 6 per cent is converted into liver glycogen, and 36 per cent is converted into body glycogen, corresponding to a recovery of absorbed sugar of 91 per cent. The values obtained for fructose are, 59 per cent oxidized, 6 per cent converted into liver glycogen, and 27 per cent into body glycogen. Of dihydroxyacetone 53 per cent is oxidized, 2 per cent deposited as liver glycogen, and 33 per cent as body glycogen.

3. After insulin injections, the utilization of absorbed sugar in the peripheral tissues (mainly muscles) is increased to such an extent that there is almost no sugar available for glycogen deposition in the liver. The diminished glycogen storage in the liver corresponds to 12 per cent of the absorbed sugar in the case of glucose, to 32 per cent in the case of fructose, and to 19 per cent in the case of dihydroxyacetone. The surplus of sugar available to the peripheral tissues during insulin action is disposed of in a different way, depending on the sugar which is fed. With glucose

and the triose it is largely increased glycogen deposition and to a smaller extent oxidation; with fructose it is mainly increased oxidation and to a lesser degree glycogen formation. A *surplus* of insulin accelerates oxidation and storage of sugar in the peripheral tissues. On the other hand, when insulin is secreted according to physiological needs, the insulin production of the pancreas is so adjusted that the utilization of sugar in peripheral tissues does not gain preponderance over that in the liver.

4. The heat production in the experiments with sugar plus insulin is slightly higher than in those with sugar alone. There is no basis for the assumption that insulin has a different effect on the O_2 intake of small laboratory animals as compared with large ones. In both types of animals the changes in O_2 consumption are small if non-convulsive doses of insulin are injected.

5. The rôle of absorption is discussed. It is believed that the following factors apply to all mammals: (*a*) selective permeability of the intestine, resulting in a different rate of absorption of isomeric hexoses and pentoses; (*b*) absorption of each sugar at a constant rate; (*c*) mutual inhibition in absorption when two different sugars or sugars and amino acids are ingested simultaneously; (*d*) reduced rate of absorption after prolonged fasting and influence of other bodily conditions on the rate of absorption; (*e*) the blood sugar curve cannot be used as an index of the rate of absorption.

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THE INFLUENCE OF THE PURINE DIURETICS ON INORGANIC PHOSPHATES OF BLOOD AND URINE.

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It has been found that glucose, pituitrin, epinephrine, insulin (1), strychnine (2), and the anesthetics (3) have a marked effect on phosphate metabolism. On further investigation it was found that other drugs exist which greatly influence inorganic blood phosphates and phosphates in the urine. This report deals specifically with the action of the purine diuretics. Bock and Iversen (4) reported a depression in the inorganic phosphates of the plasma with an increased excretion of phosphates in the urine during theophylline diuresis. In diuresis produced by administration of water the phosphates of the plasma and of the urine remained almost stationary. Havard and Reay (5) state that the rate of phosphate excretion is independent of the water rate even when, owing to copious diuresis, the urinary phosphates are below the level of the plasma phosphates. The interpretation of their findings in regard to water diuresis is somewhat confusing because of the fact that their subjects were strenuously exercising over a short period of time. Other workers (6, 7) have also studied the behavior of phosphates in the urine after different forms of diuresis, concerning themselves with the question of the absorption of phosphates in the renal tubules.

Our work which was done on dogs consisted in observing the inorganic plasma phosphates and the urine phosphates after administration of the usual purine diuretics. We also studied the sugar content of blood and urine in many of these experiments.

Methods.

Caffeine (1, 3, 7-trimethylxanthine) was given as caffeine sodium benzoate subcutaneously, intravenously, and also by mouth.

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Theobromine (3, 7-dimethylxanthine) was given by mouth in the form of diuretin (theobromine sodium salicylate). Theophylline (1, 3-dimethylxanthine) was given by mouth. Euphylline, theophylline-ethylenediamine, was given intravenously.

These drugs were administered to twenty-eight apparently normal dogs. The method of Benedict and Theis (8) was used for the determination of the inorganic plasma phosphates and the

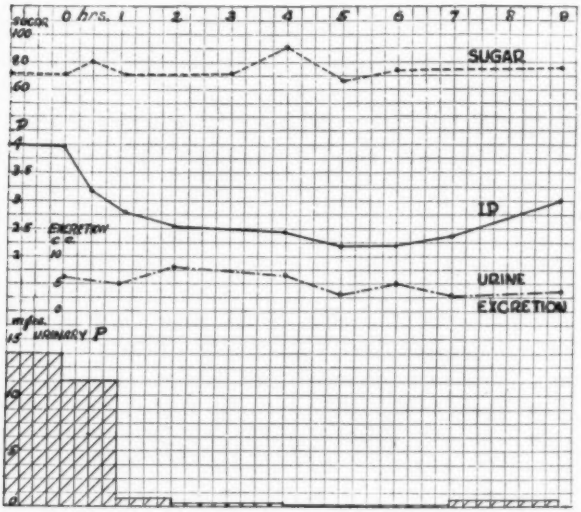

CHART 1. Showing the effect of 1 gm. of caffeine sodium benzoate, administered subcutaneously, on blood and urinary phosphates, blood sugar, and urine excretion. Height of cross-hatched areas indicates urinary phosphorus output per hour.

urine phosphates, and that of Folin and Wu (9) for the blood sugar content. The drugs were given not less than 16 hours after the last meal and plenty of water was supplied. The bladder was emptied completely by catheterization before starting the experiment and the animals were usually catheterized at hourly intervals. The urine specimens were tested for reducing substances by Benedict's qualitative reagent.

EXPERIMENTAL.

Caffeine.—Chart 1 refers to a fasting dog of 17 kilos which received subcutaneously 1 gm. of caffeine sodium benzoate. Typical diuresis could not be established in this experiment in spite of the rather large dose of caffeine administered. The blood sugar showed very little fluctuation and no reducing substances were found in the urine. Inorganic phosphates of the blood and the urinary phosphates were depressed after administration of the drug. The recovery toward normal of these constituents was very slow, and even 9 hours after administration of the drug the inorganic blood phosphates were still found to be below the normal fasting level.

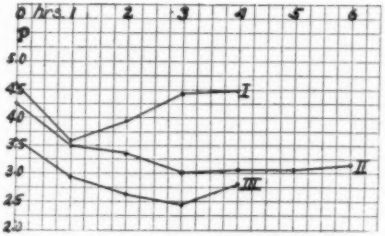

CHART 2. Changes in the plasma phosphates following the administration of caffeine sodium benzoate. Curve I, 0.5 gm. intravenously; Curve II, 0.5 gm. subcutaneously; Curve III, 1 gm. subcutaneously.

Chart 2 illustrates the effects of caffeine sodium benzoate on the inorganic phosphates of the blood after various forms of administration and different dosages. Curve I was obtained after the administration of 0.5 gm. of caffeine sodium benzoate intravenously. Curves II and III were obtained after the subcutaneous injection of caffeine sodium benzoate; 0.5 gm. of the drug was given in the experiment represented by Curve II and 1 gm. was given in that for Curve III. In Curve I, after the intravenous injection, the recovery toward normal was rather fast while Curves II and III are identical with the curve in Chart 1 except that in Curve III a tendency toward recovery appeared earlier. The hourly phosphate output in the urine was also determined in

these experiments and the findings were essentially the same as in the experiment demonstrated in Chart 1.

In the case of a highly nervous and excitable dog increased blood phosphates (from 4.4 to 5 mg.) were found 1 hour after administration of 1 gm. of caffeine sodium benzoate by stomach tube. Other than these the findings in the blood and urine phosphates were essentially the same as those in the experiments where caffeine was administered subcutaneously. Small amounts of reducing substances were found in all the urine specimens through-

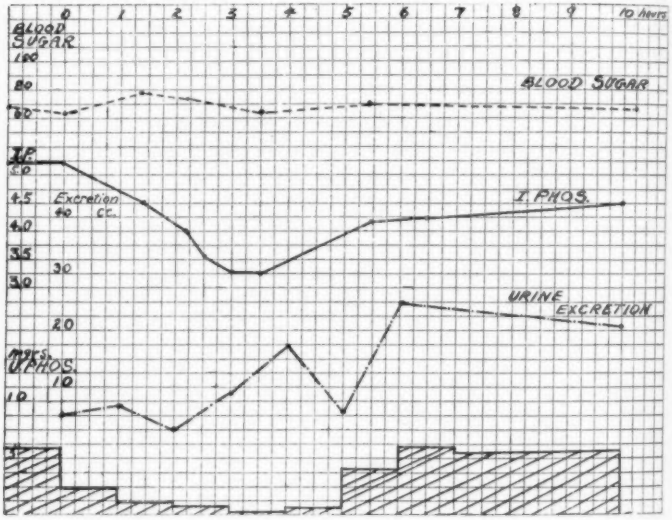

CHART 3. Results following the administration of 3 gm. of diuretin orally.

out the experiment except the last one. Fluctuations of the blood sugar content were very small, the highest blood sugar being 92 mg. 4 hours after the injection and the lowest blood sugar being 75 mg. 8 hours after the injection. Subcutaneous and oral administration of the drug produced no signs of distress in any of the experiments even after dosages as high as 100 mg. per kilo of body weight had been given.

Diuretin.—Chart 3 refers to a fasting dog of 16 kilos. 3 gm. of diuretin were administered by stomach tube. About 40 minutes after administration of the drug the animal vomited once. The drug produced a distinct diuresis but the onset was rather slow, about 3 hours after administration. None of the urines examined contained reducing substances. The variations of the blood sugar were exceedingly small. The inorganic phosphates of the blood and the urinary phosphates were markedly depressed.

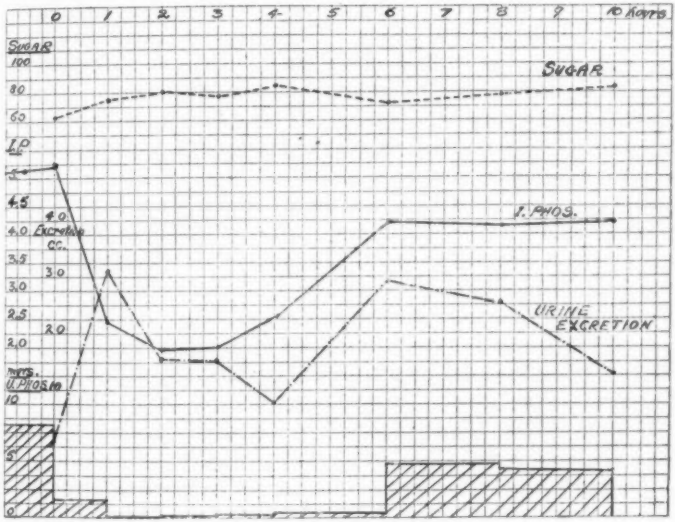

CHART 4. Results following the administration of 1.4 gm. of theophylline orally.

Theophylline.—In the experiment represented by Chart 4, 1.4 gm. of theophylline were administered by stomach tube to a dog weighing 17 kilos. The animal showed no ill effects whatever during the entire experiment. A marked diuresis was brought on by this drug. The hourly urine specimens contained no reducing substances. The blood sugar changes were of small amplitude. The inorganic phosphates of the blood decreased abruptly and similar changes were seen in the urinary phosphates.

Euphylline.—0.9 gm. of euphylline was given by vein to a dog of 16 kilos body weight. No ill effects such as vomiting or prostration were noticed throughout the experiment. A tremendous diuresis started immediately after injection. The animal produced 83 cc. of urine during the 1st hour. The blood sugar rose from a fasting level of 83 mg. to a level of 123 mg. 2 hours after injection of the drug. Later on blood sugar diminished gradually again reaching the normal level 6½ hours after administration of the drug. ½ hour after administration of the euphylline a very marked depression of the inorganic phosphates of the plasma was observed, *i.e.* from 5.2 to 3.8 mg. This depression continued until the end of the 1st hour of observation. The inorganic plasma phosphates were found to be at the low level of 2.8 mg. They remained practically at this same low level for the next hour but later on a slow but steady recovery was noticed reaching a level of 4.7 mg. 6½ hours after injection of the drug. During a further observation period of 1½ hours the inorganic phosphates of the plasma remained at the same level. The urine phosphates showed hardly any depression during the 1st hour after administration of the drug due, we think, to the marked diuresis. During the 2nd hour of the experiment the excreted urine phosphates were very low, below 1 mg. for the entire hourly output, and remained at this very low level during 2 succeeding hours. With the plasma phosphates increased to normal level the phosphate output of the urine became more abundant as in other experiments with purine diuretics.

Comparison of Quantitative Activity of Different Diuretics in Regard to Phosphate Metabolism.—After the fact was established that the different purine diuretics produce a disappearance of phosphates from blood and urine for a certain length of time a quantitative method was employed to determine the maximum possible amount of phosphates which could be retained in the tissues during a period of 2 hours after administration of a purine diuretic. For this purpose the 2 hour phosphate test was employed. This test will be described elsewhere, but on this occasion it should be mentioned that from 80 to 95 per cent of the sodium phosphates injected intravenously into dogs are excreted in the urine during the 2 hours of the experiment. In this investigation we established the normal phosphate excretion after ad-

TABLE I.

Dog No.	Weight.	Normal P per 2 hrs. (A)	P per 2 hrs. under drug influence. (B)	Difference. (A)–(B)	Plasma P before administration of drug.	Plasma P before administration of phosphates.	Plasma P at end of test.	Remarks.
	kg.	*per cent*	*per cent*	*per cent*	*mg.*	*mg.*	*mg.*	
1	16	81 80	75	5	3.36	2.59	4.10	470 mg. P injected 60 min. after 2 gm. caffeine sodium benzoate subcutaneously.*
2	15.5	81 82	77	4	4.04	3.68	4.19	470 mg. P injected 50 min. after 1 gm. caffeine sodium benzoate subcutaneously.
3	9.5	86 89	61	26	3.38	3.04	3.29	470 mg. P injected 60 min. after 1.5 gm. diuretin by mouth.
4	17	88 84	70	16	3.72	3.45		" "
5	14.5	82 80	61	20	3.98	3.50		470 mg. P injected 60 min. after 1.7 gm. theophylline by mouth.
6	14.0	92 86	64	25	3.90	2.08	3.56	470 mg. P injected 35 min. after 0.98 gm. euphylline intravenously.
6	14.0	92 86	58	31	4.21	2.13		" "
2	15.5	81 82	48	33	4.37	2.49	3.82	470 mg. P injected 60 min. after 0.98 gm. euphylline intraveneously.
7	13.5	81 79	57	23	4.67	2.08	3.68	470 mg. P injected 40 min. after 0.98 gm. euphylline intravenously.
7	13.5	81 79	48	32	3.98	1.95		470 mg. P injected 70 min. after 0.98 gm. euphylline intravenously.†

* Phenolsulphonephthalein output under drug influence, 65 per cent.
† Phenolsulphonephthalein output under drug influence, 60 per cent.

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ministration of 470 mg. of phosphorus as sodium phosphates (pH = 7.3) in a number of normal dogs. Then, after a period of rest of at least a week, purine diuretics were adminstered and the phosphate test was employed again to determine the amount of phosphates excreted during the most effective period of drug influence.

In order that no doubt may exist in regard to a possible impairment of kidney function through the use of purine diuretics we injected in a few instances 6 mg. of phenolsulfonephthalein in conjunction with the phosphates.

In Table I the findings are summarized. On every animal phosphate tests were done at least twice to determine the normal excretion before the drugs were given. In the two experiments with caffeine the depression was 4 and 5 per cent. In the two experiments with diuretin the depression was 16 and 26 per cent. Only one experiment was done with theophylline which showed a diminution of excretion of 20 per cent. Five experiments were done with euphylline showing an average depression of 29 per cent, the maximum depression being 33 per cent and the minimum depression 23 per cent. The plasma phosphates as determined before injection of the sodium phosphate solution showed in each case a depression due to the action of the purine diuretics previously given. At the end of the phosphate test plasma phosphates ranged around normal, being somewhat above normal in cases with slight retention and somewhat below normal in cases with strong retention in the tissues.

DISCUSSION.

The inorganic phosphates of the plasma of a moderately fasting dog may be considered at a stable level for at least 10 hours. Moderate bleeding, 10 to 20 cc. per hour, has no effect on the inorganic phosphate level of the plasma; further, for example, the injection of 5 cc. of distilled water either in the vein or subcutaneously or infusion of 20 cc. of a 20 per cent solution of sodium thiocyanate, has no effect. The administration of therapeutic doses of caffeine, theobromine, or theophylline had, in our experiments, a qualitative uniform effect on the inorganic phosphates of the blood and on the phosphate excretion in the urine. No special attention has been paid to the alterations in the concentration of

the blood plasma. In a few instances a slight concentration of the blood plasma after the administration of the purine bases was noticed in accordance with observations of other workers. Such variations, of course, would not appreciably alter the chemical observations.

The diurnal changes in the phosphate output as observed in fasting dogs and also the variations due to a demand for phosphates in the excretion of acids have been obscured entirely by the administration of these four mentioned drugs of the purine series. Also there always occurred a very marked depression of the urinary phosphates, and the excretion of phosphates by the kidneys seemed to run roughly parallel to the concentration of the phosphates in the plasma. So far only in one experiment have any irregularities occurred, namely, the experiment with caffeine in an excited dog. However, we believe that the increased inorganic phosphate level of the plasma as found 1 hour after administration of caffeine was mostly due to the momentary excitement, fear, and struggling of the animal because the urinary output of phosphates did not show a corresponding increase. However, in this experiment the inorganic phosphates of the blood followed the same course as in the other experiments except for the fact that the highest depression was markedly delayed.

Our experiments with theophylline corroborate the results of Bock and Iversen in regard to the depression of inorganic phosphates of the blood after administration of this drug but contradict their statement that the phosphate excretion is increased under the action of this drug. Bock and Iversen's work was done on unanesthetized rabbits. While there is still a possibility that the rabbit reacts differently than does the dog in this respect, we call attention to the possible effects of psychic factors and trauma, as detailed in our work on phosphates in anesthesia (3).

The intensity of action of the different purine diuretics in regard to phosphate metabolism can hardly be made out from the blood and urine curves as obtained after the administration of these drugs. The excretion of urine phosphates soon reaches a very low level of practically zero while the phosphates in the plasma cannot be depressed below a certain level in spite of the heaviest and repeated doses of diuretics. Concerning the time over which the different diuretics act, the mode of administration sometimes

plays a very important rôle, especially in the case of caffeine. The effects of an intravenous administration of caffeine on inorganic plasma phosphates are fleeting, while caffeine given subcutaneously or by mouth depressed the inorganic phosphates for many hours (Charts 1 and 2). It seems as if caffeine administered intravenously becomes inactive in a short period of time while caffeine administered orally or subcutaneously is released slowly into the blood stream simulating a prolonged action. In contrast to caffeine in this respect are theophylline and euphylline, the former administered orally, the latter intravenously. We were impressed by the similarity of the curves as they were obtained after administration of the same principle by mouth and by vein.

In order to obtain any quantitative information about the relative activity of the different purine diuretics and also for purposes of comparison with other drugs having a similar action on phosphate metabolism, we applied the 2 hour phosphate test. From these findings it is evident that caffeine is by far the weakest agent affecting phosphate metabolism while the other purine diuretics are of about similar strength. Diuretin is perhaps somewhat less effective than theophylline or euphylline. We also found in this connection that a relationship seems to exist between the specific diuretic action of the purine derivatives used in our experiments and their effect upon phosphate retention. Thus it was noticed that the greater the degree of diuresis produced by the drug the greater the degree of phosphate retention. In this respect diuretin, theophylline, and euphylline were found to have a stronger effect than caffeine, the former two drugs causing a more consistent and profuse diuresis with a correspondingly high phosphate retention.

In comparison with other drugs it might here be stated that the purine diuretics are not the most effective ones. The effect of insulin is yet more marked and a retention of 60 per cent of the phosphates is easily produced with a therapeutic dose of this hormone.

As intimated before the possibility of kidney disorder was disproved by the use of phenolsulfonephthalein. A normal excrétion of the dye was in each case obtained though combined with the injected phosphates.

Considering the possible relationship between phosphate and

carbohydrate metabolism and because the information from the literature regarding sugar metabolism under purine diuretics is not conclusive, we also determined the sugar content of the blood and urine in our experiments. We found that a rise of the blood sugar content above 100 mg. per 100 cc. could only rarely be obtained after the administration of a rather large dose of euphylline. In other experiments with similar or smaller doses of euphylline the blood sugar alterations were about the same as with caffeine, diuretin, and theophylline; *i.e.*, an increase of about 15 mg. of sugar per 100 cc. of blood. From these experiments we feel justified in saying that the changes in the blood sugar content produced by the administration of therapeutic doses of the four investigated diuretics are insignificant. Reducing substances in the urine were only found after administration of caffeine by the intestinal route.

SUMMARY.

1. The following purine diuretics: caffeine sodium benzoate, diuretin, theophylline, and euphylline depress inorganic blood phosphates as well as the phosphate excretion in the urine.

2. The phosphate demand in the tissue under the action of these drugs has been measured with the phosphate test. By this test it has been found that caffeine is much weaker in its action than the other purine diuretics.

3. The studied purine diuretics have little or no influence on the blood sugar of normal animals. Reducing substances in the urine were observed in the case of caffeine administered by mouth.

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THE STRUCTURE OF TETRAMETHYL-γ-METHYL-MANNOSIDE.

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Our interest in the study of the so called γ-sugars[1] is due to a desire to correlate the oxidic structure of a sugar with its physical and chemical properties.

Under the name of γ-sugars are classified all those forms which show a chemical behavior different from that of the common forms. In certain respects the thus far described γ forms resemble each other, first, in the high velocity of the reaction of substitution of the hydrogen of the hydroxyl on carbon atom (1) and secondly, in the high rate of hydrolysis of their glucosides.

In the details of their chemical behavior the γ forms of different sugars show among themselves very marked differences.

In this place we shall discuss only two sugars, namely γ-glucose and γ-mannose. γ-Methylglucoside and γ-methylmannoside resemble each other in the velocity of their hydrolysis, but in other respects similarity is lacking. The α and β forms of the methylated γ-methylglucosides are readily interconvertible, whereas, the interconversion of the corresponding forms of mannose has not yet been accomplished. γ-Methylglucoside is very readily methylated, whereas, difficulty is encountered in the complete methylation of γ-methylmannoside. Furthermore, γ-methylmannoside has a tendency towards shifting its ring structure, a property thus far not observed to occur to the same extent in other glucosides.

Are these differences caused by differences in the ring structures of the two γ-glucosides or by differences in their configurations? Be-

[1] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1927, lxxiv, 701.

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cause of these considerations, we undertook to determine the ring structure of the methylated γ-methylmannoside.

Tetramethyl-γ-mannose was therefore oxidized by the Willstätter and Schudel method following the details worked out by Goebel. On oxidation of 3.0 gm. of tetramethyl-γ-mannose, 0.300 gm. of <1, 4> tetramethylmannonic lactone was obtained. The lactone was identical in its melting point, its rotation, and its composition, with the one obtained on methylation of mannonic lactone.[2]

From this observation the conclusion is warranted *that tetramethyl-γ-methylmannoside at least in part consists of a <1, 4> mannoside.*

In view of the fact that the ring structure of the γ-methylmannoside is readily isomerized, it is as yet not absolutely certain that the structure of unmethylated γ-mannoside is identical with that of the methylated γ-methylmannoside, nor is it as yet certain that the tetramethyl-γ-mannoside is not a mixture of several ring isomers. Work on the solution of these problems is now in progress.

EXPERIMENTAL.

γ-Methylmannoside was prepared according to the directions of Irvine and Burt,[3] and was further methylated by the silver oxide and methyl iodide method. The product of the first two methylations was fractionated and only the low boiling fraction was used for further methylation.

Tetramethyl-γ-Methylmannoside.—The trimethyl-γ-mannoside was methylated by silver oxide and methyl iodide, a considerable excess of the latter reagent being used. This prevented excessive loss of material. 25 gm. of trimethylmannoside were dissolved in 50 cc. of methyl iodide, and 125 gm. of silver oxide and 200 gm. of methyl iodide were added in small portions as usual. After each methylation, the methylated product was fractionated, the lowest boiling fraction set aside, and the higher fraction submitted to further methylation. All lower boiling fractions were then com-

[2] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1924, lx, 167.
[3] Irvine, J. C., and Burt, W., *J. Chem. Soc.*, 1924, cxxv, 1343.

bined and remethylated. The final product distilled at 105°, p = 0.3 mm., and analyzed as follows:

6.322 mg. substance: 12.100 mg. CO_2 and 4.765 mg. H_2O.
0.0979 gm. " : 0.4674 gm. AgI.
Calculated. C 52.8, H 8.8, OCH_3 62.
Found. " 52.2, " 8.4, " 63.

It had a specific rotation in methyl alcohol of

$$[\alpha]_D^{20} = \frac{+0.99° \times 100}{1 \times 4.00} = +24.7°.$$

Oxidation of Tetramethyl-γ-Mannose.—The γ-mannoside, 5 gm., was hydrolyzed with 0.1 N HCl for 90 minutes in boiling water. The hydrochloric acid was neutralized with barium carbonate and the sugar isolated as usual. The yield was 3 gm. This was oxidized by the method of Willstätter and Schudel, modified by Goebel[4] as follows: The sugar dissolved in a little water was added to 170 cc. of 0.3 N iodine in barium iodide. To this solution were added, with constant stirring, 256 cc. of 0.4 N $Ba(OH)_2$. After all of the alkali had been added, the solution was allowed to stand for 15 minutes. It was then acidified with 40 cc. of 25 per cent sulfuric acid and the sulfuric acid removed with 40 gm. of lead carbonate. The solution was centrifuged, and the precipitate washed with water several times, and finally extracted with alcohol. The solutions were all combined and concentrated under reduced pressure to a small volume. The solution was then treated with a slight excess of silver sulfate and, after filtering, the silver was removed by hydrogen sulfide. The filtrate was concentrated and the sulfuric acid removed quantitatively with a solution of barium hydroxide. The solution was then concentrated to a syrup under diminished pressure, the residue was extracted with ether, and the extract was dried over anhydrous sodium sulfate.

On slow evaporation of the ethereal solution a crystalline deposit formed. It was filtered by suction and washed with small portions of a solution consisting of equal parts of ether and petrolic ether. In this manner 0.300 gm. of needle-like crystals was

[4] Goebel, W. F., *J. Biol. Chem.*, 1927, lxxii, 809.

collected. Without recrystallization the substance melted at 107–108° and analyzed as follows:

5.105 mg. substance: 9.645 mg. CO_2 and 3.515 mg. H_2O.
0.1014 gm. " : 0.4076 gm. AgI.
Calculated. C 51.2, H 7.7, OCH_3 53.0.
Found. " 51.5, " 7.7, " 53.1.

In cold water it had a rotation of

$$[\alpha]_D^4 = \frac{+ 1.12° \times 100}{1 \times 1.728} = + 64.8°.$$

THE FORMATION OF SUGAR FROM FATTY ACIDS IN THE DEPANCREATIZED DOG INJECTED WITH EPINEPHRINE.

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(Received for publication, November 2, 1927.)

From observations based mainly on D:N ratios of phlorhizinized dogs, Lusk has concluded that protein is the source of the new sugar in the diabetic organism and that the fatty acid molecule does not suffer such a conversion. His main contention is the constancy of this ratio which for phlorhizinized dogs he found to be 3.65 contrasting with that in the totally depancreatized dog which Minkowski found to be 2.85. In recently published data from this laboratory, it was shown that in totally depancreatized dogs, this ratio is not constant, but depends upon the nutritional condition of the animals. Thus, it varies in different depancreatized dogs, being in general higher in fat than in lean ones, and it varies decidedly in the same animal according to its nutritional condition when the diabetes is brought on by discontinuing insulin. Neither does it become stabilized at any definite level, but steadily falls (Chaikoff, Macleod, *et al.*, 1925; Macleod and Markowitz, 1926; Chaikoff, 1927).

As a matter of fact, if the D:N ratio observed on the 5th day after removal of insulin and food from depancreatized dogs be corrected for all the available carbohydrate, such as glycogen of the liver and muscles and the known sources of carbohydrate, such as glycerol, the resulting ratio is still in excess of 3.65, which according to Lusk, is the level at which protein is converted to sugar in the phlorhizinized animal (Chaikoff, 1927). In the light of the present day teaching such high ratios are indicative of a conversion of fat to carbohydrate.

The hypothesis that fatty acid is transformed to carbohydrate is considered to receive strong support from observations on the R.Q. of animals whose livers have been completely removed or have been eliminated from the circulation. Porges (1910) and Porges and Salomon (1910) found in depancreatized dogs that following removal of the liver from the circulation, the R.Q. rose to unity or close to unity. They concluded that the diabetic organism retains the power to oxidize carbohydrate, and that the liver is essential for gluconeogenesis from fat and protein. In recent years these older observations have been confirmed by Burn and Dale (1924) and by Markowitz (1928), the latter using Mann's technique for complete extirpa-

tion of the liver. The validity of drawing conclusions from such preparations has been called in question by Murlin, Edelmann, and Kramer (1913-14) who, in repeating the work of Porges and Salomon, found that coincident with the rise in R.Q. the CO_2 of the blood fell, whereas in those animals in which the R.Q. did not rise, the blood CO_2 was maintained at the normal level. Although it is conceivable that the loss of CO_2 from the blood might in part explain the rise in R.Q. during the early periods following hepatectomy, it is difficult to perceive how such a mechanism could persist so as to bring about quotients close to unity during the 2nd or 3rd hour after removal of the liver.

Krogh and Linhard (1920) found that fat is used in muscular activity at an increased expenditure of energy of 11 per cent as compared with carbohydrate. In the light of the results of Meyerhof, who has shown clearly that muscular contraction in the isolated frog muscle is concerned solely with glycogen as a fuel, it seems highly probable, as these workers suggest, that this extra expenditure of energy in the case of fat, is due to the conversion of the latter to carbohydrate before its utilization in the muscles. The most recent and significant work on this point is that of Furusawa (1925), who found that the R.Q. of the excess metabolism of a short period of exercise is unity, even when the subject has lived on a diet of fat and protein for several days and has a resting quotient of 0.71.

In the present investigation strong evidence for the conversion of fatty acid into carbohydrate has been obtained, by finding that when epinephrine is administered to depancreatized dogs on the 4th day following withdrawal of insulin and food, much larger amounts of glucose are excreted than can be accounted for as coming from the known possible sources of carbohydrate, including protein.

Methods.

Following complete extirpation of the pancreas the dogs received insulin twice daily and were placed on a diet of raw meat, raw pancreas, and sugar. When the wound had completely healed and the animal was in good nutritional condition, food and insulin were discontinued. After the 2nd day of fasting, urine was collected every 12 hours, the animals being catheterized and the bladder carefully washed with distilled water. Nitrogen was determined by the Kjeldahl method, glucose by the Shaffer-Hartmann method (1921), ketones by the Van Slyke method (1917), and phosphates by the Briggs modification (1922) of the Bell-Doisy method. The caloric expenditure of the animals was obtained by the Zuntz-Schumburg calculation of the oxygen consumption, which was

measured in a respiratory chamber of the Benedict type. Glycogen was determined by Pflüger's method. Lactic acid of the blood was determined by the permanganate oxidation on the tungstic acid filtrate after removal of interfering substances with copper sulfate and lime.

TABLE I.

Dog F, weight 6.8 kilos. Food and insulin discontinued May 23, 8.00 a.m. (This dog fasted twice previously for periods of 4 days each.)

Date.	Period.	Glucose.	Nitrogen.	D:N	Inorganic phosphate.	R.Q.	O_2 consumed per kilo per hr.	Calories per kilo per 12 hrs.	Remarks.
1927		gm.	gm.		mg.		cc.		
May 25	First 12 hr., 3rd day.	16.80	2.62	6.4	241	0.683	604*	34	
" 25	Second 12 hr., 3rd day.	11.05	2.46	4.5	177				
" 26	First 12 hr., 4th day.	42.41	3.16	13.4	340	0.747	874†	50	4 doses of 1 cc. 1:1000 adrenalin given over 12 hr. period.
" 27	Second 12 hr., 4th day.	14.33	2.56	5.6	352				

* Measured over first 3 hours of period.

† Measured over first 3 hours of period after first dose of adrenalin.

Results.

The results of Dog F are presented in Table I. During the first 12 hours of the 4th day of fasting, the animal received 1 cc. of 1:1000 epinephrine every 3 hours. During this period the animal excreted 42.4 gm. of glucose, as compared with 11.05 gm. in the previous 12 hours.

In a number of observations made by one of us (Chaikoff, 1927) it was found that the glucose eliminated during the last 12 hours of the 3rd day of fasting in depancreatized dogs, did not vary by any significant amount from that excreted during the first 12 hours

of the 4th day of fasting. For convenience the results of five dogs of varying sizes are tabulated below.

Fasting period.	Glucose excretion, gm.				
Second 12 hrs. of 3rd day.....	15.6	4.10	11.2	6.40	9.20
First 12 " " 4th "	12.0	5.62	12.1	7.15	6.80
Difference.....	−3.6	+1.5	+0.9	+0.75	−2.4

It will be observed that when the glucose excretion was greater during the first 12 hours of the 4th day than on the last half of the 3rd day, the increase was only slight. When less was excreted during the 4th day period, it proved to be in those animals in which the glucose excretion fell rapidly throughout the fasting period.

With this evidence in mind we may assume that if epinephrine had not been administered, approximately 11.1 gm. of glucose would have been excreted by Dog F during the first 12 hours of the 4th day. We have then to account for 42.4 − 11.1 = 31.3 gm. of extra glucose excreted under the influence of epinephrine, for which the following possible sources are to be considered.

1. Liver Glycogen.—The maximum amount of glycogen found in the livers of totally depancreatized dogs at the end of the 3rd day of fasting was 0.35 per cent (Chaikoff, 1927). The weight of the liver of Dog F was 340 gm. Assuming the total conversion of the liver glycogen to glucose in the animal and its excretion in the urine, not more than 1.2 gm. of urinary glucose can be thus accounted for.

2. Muscle Glycogen and Lactic Acid.—Several investigators in recent years have established definitely that muscle glycogen cannot contribute directly to the sugar of the blood. Thus Mann and Magath (1925) showed that those methods used to induce hyperglycemia in normal animals (asphyxia and epinephrine) were without effect on the blood sugar of dogs in which the liver had been removed, although Bollman, Mann, and Magath (1925) found considerable amounts of glycogen in the muscles of hepatectomized dogs suffering from hypoglycemia. These observations have been fully confirmed in this laboratory by Soskin (1927, *a*). In eviscerated spinal cats Best, Hoet, and Marks (1926) found that the muscle glycogen remained constant over periods of about 5 hours during which time the blood sugar fell steadily to low levels.

The evidence of all these workers points to the conclusion that once the sugar of the blood has become synthesized to muscle glycogen it cannot again leave the muscle in the form of glucose. There remains the possibility, however, that excess lactic acid produced in very severe muscular exercise by the anaerobic breakdown of muscle glycogen, may, after conversion in the liver, reach the blood as glucose. Bierry and Gatin-Gruzewska (1905) have reported a fall in muscle glycogen as a result of the administration of epinephrine. We have been able to confirm this in the standard white rat, but Choi (1928) has demonstrated in the decapitate cat that muscle glycogen disappears, significantly only when the epinephrine causes spasms. Since the disappearance of muscle glycogen is not due to its loss into the blood as glucose, the possibility that increased oxidation might account for its disappearance following epinephrine, has to be considered. Although this would seem to be borne out by the fact that epinephrine raises both the R.Q. and O_2 consumption of normal animals fasted for 24 hours, it must be remembered that epinephrine fails to raise the O_2 consumption after hepatectomy (Soskin 1927, *b*).

It seemed important to ascertain whether excess of lactic acid is discharged into the blood following administration of epinephrine to eviscerate preparations, and the results of such experiments are shown in the following protocols.

Blood Sugar and Lactic Acid in Eviscerated Dog (Dog A) Following Epinephrine.

Time.	Procedure.	Blood sugar.	Lactic acid.	Remarks.
p.m.		*per cent*	*per cent*	
2.00–2.30	Evisceration.			
2.45		0.124	0.076	Animal restless.
3.15		0.097	0.030	
	1.5 cc. 1:1000 adrenalin subcutaneously.			
3.30		0.091	0.018	
4.00		0.067	0.023	
4.30		0.054	0.012	

Muscle glycogen, left hind leg 0.88 per cent, left front leg 1.16 per cent.

Besides confirming the conclusion of previous investigators that epinephrine can neither raise the blood sugar in hepatectomized

animals nor deplete the stores of muscle glycogen, the results show that no lactic acid appears in the blood as a result of the injection of epinephrine, which might have muscle glycogen as its source. We may, therefore, conclude that *muscle glycogen is not a source of the extra sugar eliminated in the urine by the organism under the influence of epinephrine.*

3. Protein.—From his investigations on phlorhizinized dogs Lusk concluded that 58 per cent of the protein may be converted to glucose. On the other hand, if we calculate the maximum amount of sugar which might be formed from protein on the assumption that all of the carbon of the protein molecule that is not in the urine or feces is thus converted, the following results are obtained.

100 gm. of dried meat, free of fat, contain (Loewy, 1926): 52.38 gm. of C and 16.65 gm. of N of which 9.41 gm. of C and 16.28 gm. of N are eliminated in the urine and 1.47 gm. of C and 0.37 gm. of N are eliminated in the feces.

This leaves 41.5 gm. of carbon for oxidative purposes, which, if completely transformed to sugar could yield 104 gm. of glucose. On this basis 16.28 gm. of urinary glucose would indicate the formation of 104 gm. of glucose, which is the maximum amount of carbohydrate derivable from protein, or 1 gm. of urinary nitrogen indicates the formation of 6.4 gm. of glucose. Although it is highly improbable that all the carbon of protein could be converted to glucose, it is important to observe that even if it were, only a small proportion of the extra glucose excreted as a result of the injection of epinephrine could be thus accounted for. Thus, in Dog F the nitrogen in the urine during the epinephrine period rose by 0.70 gm. At most then, this could account for $0.70 \times 6.4 = 4.5$ gm. of glucose.

To possess any significance for the above calculation the nitrogen excreted in the urine must represent the breakdown of protein which is occurring in the body during the period in which it is measured. That no nitrogen retention had occurred during the period in which epinephrine was injected in Dog F is shown by the fact that the excretion of nitrogen in the period following the injection fell to about the same level as that of the 12 hours immediately preceding it.

4. Glycerol.—The total caloric expenditure of the animal during

the period in which epinephrine was injected was 340 calories. Assuming that only fat is burned by the diabetic organism, this would correspond to the utilization of $\frac{340}{9.4}$ = 36 gm. of fat. The glycerol content of this amount of fat is 3.6 gm. which might be converted to glucose during the first 12 hours of the 4th day of fasting.

The total amount of glucose that could be accounted for by liver glycogen, protein, and glycerol in Dog F is, therefore, 1.20 + 4.5 + 3.6 = 9.3 gm., which leaves unaccounted for 31.3 − 9.3 = 22.0 gm. of glucose. We are, therefore, forced to conclude that at least 22.0 gm. of glucose must have been derived from fatty acids.

The maximum amount of glycogen found by one of us (Chaikoff, 1927) in muscles of depancreatized dogs at the end of the 3rd day of fasting was 0.47 per cent. In Dog F, even if we make the entirely unwarranted assumption that this glycogen might be converted to, and excreted as glucose, it would amount to[1] $\frac{40}{100} \times \frac{6.8}{1} \times \frac{0.47}{100} \times 1000$ = 12.8 gm., still leaving 22.0 − 12.8 = 9.2 gm. of glucose unaccounted for by all known sources of carbohydrate, outside of fatty acids.

The above observations have been repeated on six other animals, and the results are shown in Tables II to VII.

Dog J (Table II).—It will be observed in Table II that two periods of fasting were carried out on Dog J, and that the D:N ratios on the 3rd day of the second period of fasting were in general the same as the D:N ratios of the corresponding day of the first period of fasting.

Epinephrine was given during two periods of 6 hours each, and the assumption has been made that all the protein represented by the nitrogen excretion of each period was completely converted to glucose and excreted as such.

During the first 6 hours that epinephrine was administered 31.5 gm. of glucose were excreted. Protein might account for 1.47 ×

[1] Assuming the muscles to constitute 40 per cent of the body weight.

6.4 = 9.4 gm. of glucose; and using the same values as in Dog F (Table I), liver glycogen will account for 1.6 gm. and glycerol for 2.4 gm., or a total of 13.4 gm. The carbohydrate derived from fatty acid in Dog J in 6 hours of epinephrine administration will, therefore, be 18.1 gm. (The total glycogen present in the muscles of this animal was calculated on the same basis as for Dog F to be 16.9 gm.)

TABLE II.

Date.	Period.	Glucose.	Nitrogen.	D:N	Phosphates.	Remarks.
A. Dog J, weight 9.0 kilos. Food and insulin discontinued Aug. 19, 9.00 a.m. Second fasting.						
1927		*gm.*	*gm.*		*mg.*	
Aug. 21	First 12 hr., 3rd day.	11.6	1.75	6.6	198	
" 22	Second 12 " 3rd "	12.4	1.81	6.8	168	
" 22	First 12 " 4th "	Urine lost.				
" 23	Second 12 " 4th "	6.4	1.75	3.6	187	
" 23	First 12 " 5th "	7.0	1.71	4.1	168	
B. Dog J, weight 9.0 kilos. Food and insulin discontinued Sept. 3, 9.00 a.m. Third fasting.						
Sept. 5	First 12 hr., 3rd day.	17.3	2.35	7.4	303	
" 6	Second 12 " 3rd "	12.9	2.24	5.7	203	
" 6	First 6 " 4th "	31.5	1.47	21.5	85	2 doses of 1 cc. adrenalin, 3 hrs. apart.
" 6	Second 6 " 4th "	21.2	1.70	12.5	385	2 doses of 1 cc.
" 7	" 12 " 4th "	11.9	3.54	3.1	480	1:1000 adrenalin
" 7	First 12 " 5th "	4.8	1.78	2.7	212	3 hrs. apart.

Since we can more than account for all the glycogen stores, even including muscle glycogen, by the glucose excreted during the first 6 hours of the 12 hour epinephrine period, we may disregard these sources as contributing factors for the 21.2 gm. of glucose which was excreted during the second 6 hours in which epinephrine was administered. The D:N ratio during this period was 12.5, which is twice that obtained supposing the carbon of protein were completely converted to glucose. Of the 21.2 gm. of glucose excreted, protein might account for 1.7 × 6.4 = 10.9 gm., and

glycerol for 2.4 gm. of glucose, or a total of 13.3 gm. of glucose, leaving 7.9 gm. of glucose which cannot be accounted for as derived from any other source than that of fatty acids.

Dog C (Table III).—Using a similar method of calculation as for Dog F, it will be observed in Dog C (Table III, B) that the extra glucose excreted under the influence of epinephrine was 36.4 − 13.6

TABLE III.

Date.	Period.	Glucose.	Nitrogen.	D:N	Total ketone bodies.	Remarks.
A. Dog C, weight 6.3 kilos. Food and insulin discontinued May 7, 9.00 a.m.						
1927		*gm.*	*gm.*		*gm.*	
May 9	First 12 hr., 3rd day.	27.0	3.54	7.6		
" 10	Second 12 " 3rd "	24.2	3.84	6.3		
" 10	First 12 " 4th "	36.8	4.69	7.8		4 doses each of 0.4 cc. 1:1000 adrenalin given over 12 hr. period.
" 11	Second 12 " 4th "	22.3	4.00	5.5		
B. Dog C, weight 6.3 kilos. Food and insulin discontinued May 15, 9.00 a.m.						
May 18	Second 12 hr., 3rd day.	15.2	3.69	4.1	0.928	
" 19	First 12 " 4th "	13.6	3.53	3.8	1.037	
" 19	Second 12 " 4th "	36.4	3.23	11.3	1.580*	4 doses each of 1 cc. 1:1000 adrenalin given over this 12 hr. period.

* Dog killed for muscle and liver glycogen.

= 22.8 gm. This animal was killed at the end of the epinephrine period and 0.10 per cent glycogen was found in the liver. As the maximum amount of glycogen in the livers of untreated depancreatized dogs at the end of the 3rd day of fasting was found in previous observations to be 0.35 per cent, the loss in preformed liver glycogen caused by epinephrine in Dog C was $\frac{0.25}{100} \times 350$

= 0.87 gm. Protein could not have contributed to the extra glucose excreted, since the urinary nitrogen during the period in which epinephrine was injected fell from 3.5 to 3.2 gm. The O_2 consumption of this animal was not measured, but assuming it to have been the same per kilo of body weight as for a dog of similar size (Dog F, Table I), then $\frac{50 \times 6.3}{9.4 \times 10} = 3.3$ gm. of urinary glucose might have been derived from glycerol, so that taking glycogen and glycerol together 3.3 + 0.87 = 4.17 gm. of glucose can be accounted for. The glucose obtained by the animal at the expense of fatty acid was at least 22.8 − 4.17 = 18.6 gm.

Even if we make the entirely unwarranted assumption that muscle glycogen was excreted as glucose in the urine, this would only account for $6300 \times \frac{40}{100} \times \frac{0.47}{100} = 11.8$ gm., still leaving 6.8 gm. of glucose to account for.

In Table III, A, the effect of administration of small doses of epinephrine on sugar and nitrogen excretion is shown. Although the D:N ratio rose to 7.8, the result is not highly significant from the present point of view, since of the 12.6 gm. of extra glucose eliminated in the urine due to the influence of epinephrine, 9.9 gm. may be accounted for by the methods of calculation used above. This experiment is of significance in connection with the results obtained by Eppinger *et al.* (1908), Ringer (1910), and others.

Dog B (*Table IV*).—The liver of this animal was not weighed, but assuming it to be 5 per cent of the total body weight, it would be 310 gm. The liver would thus contain $\frac{310 \times 0.35}{100} = 1.1$ gm. of glycogen. The nitrogen excretion increased by 0.95 gm. so that protein might possibly have contributed 6.4 × 0.95 = 6.1 gm. of glucose. Assuming for this dog the same caloric expenditure as for a dog of similar size (Dog F, Table I), glycerol might account for $\frac{6.2 \times 50}{9.4 \times 10} = 3.3$ gm. of glucose. The glucose derived from fatty acids, therefore, was at least 23 − (6.7 + 1.1 + 6.1 + 3.3) = 5.8 gm.

Dog I (*Table V*).—Dog I (Table V) was subjected to two periods of fasting with an interval of 6 days intervening, and it will be ob-

TABLE IV.

Dog B, weight 6.2 kilos. Food and insulin discontinued April 28, 9.00 a.m.

Date.	Period.	Glucose.	Nitrogen.	D:N	Total ketone bodies.	Remarks.
1927		*gm.*	*gm.*		*gm.*	
May 1	Second 12 hr., 3rd day.	7.6	1.66	4.6		
" 2	First 12 " 4th "	6.7	1.65	4.1	0.168	
" 2	Second 12 " 4th "	23.0	2.60	8.9	0.567*	4 doses each of 0.3 cc. 1:1000 adrenalin given over this 12 hr. period.

* Dog found dead May 3., *viz.*, 24 hours after last urine was taken.

TABLE V.

Date.	Period.	Sugar.	Nitrogen.	D:N	Phosphates.	Total ketone bodies.	Remarks.
A. Dog I, weight 14.0 kilos. Food and insulin discontinued July 25, 9.00 a.m.							
1927		*gm.*	*gm.*		*mg.*	*gm.*	
July 26	First 12 hr., 2nd day.	42.0	2.77	15.2	265	0.052	
" 27	Second 12 " 2nd "	22.2	2.33	9.5	257	0.052	
" 27	First 12 " 3rd "	23.2	3.78	6.1	404	0.370	
" 28	Second 12 " 3rd "	16.3	3.69	4.4	410	0.970	
" 28	First 12 " 4th "	26.0	6.17	4.2	390	2.56	
B. Dog I, weight 14.0 kilos. Food and insulin discontinued Aug. 3, 9.00 a.m.							
Aug. 5	First 12 hr., 3rd day.	44.5	2.11	21.1	282		
" 6	Second 12 " 3rd "	23.2	2.36	9.8	297		
" 6	First 12 " 4th "	38.4	0.87	44.1	189		4 doses of 1 cc. 1:1000 adrenalin every 3 hrs. over 12 hr. period.
" 7	Second 12 " 4th "	16.2	0.71	22.9	486		
" 7	First 12 " 5th "	11.6	1.80	6.4	367		

served that the D:N ratios on the 3rd day of fasting in Table V, A were 6.1 and 4.4, whereas in Table V, B they were 21.1 and 9.8 respectively. A similar fact was observed in another dog, Dog H (see Table VI). The D:N ratios on the 3rd day of the first period of fasting were 10.8 and 4.4, whereas during the second period on the 3rd day, they were 19.5 and 13.3.

When epinephrine was administered (Table V, B) during the first 12 hours of the 4th day, the glucose excreted was 38.4 gm. and the D:N ratio was 44. This extremely high ratio is in large part to be accounted for by a marked depression in the nitrogen elimination during this period (*viz.*, 0.87). In previous computations we have deducted from the glucose excreted during the epinephrine period, first, the glucose excreted during the previous 12 hours, and second, when an increase in nitrogen excretion had occurred, the glucose that might have been derived from the increased breakdown of protein. In the present case such a calculation is obviously impossible, nevertheless we have allowed the maximum amount of glucose which might have been derived from protein as being the same as that of the preceding period. If we assume that all the liver glycogen was excreted as glucose, this source would account for 2.5 gm. of glucose, and if we assume the caloric expenditure per kilo of body weight to be the same as for Dog F (Table I), glycerol could have contributed 7.5 gm. of glucose. This leaves 5.2 gm. of glucose unaccounted for, which must have had as its source fatty acids. In this animal a D:N ratio of 23 was obtained during the second 12 hours of the 4th day of fasting, when no epinephrine was administered. Since the carbohydrate derivable from all known sources had already been taken into account by the glucose excreted during the preceding 12 hours, this high ratio in itself must be regarded as strong evidence for the conversion of fat to carbohydrate.

Dog H (Table VI).—This animal was subjected to two periods of fasting and in each case epinephrine was administered during the 24 hours of the 4th day of fasting. In Table VI, A the extra glucose eliminated under the influence of adrenalin was $28.8 - (9.27 \times 2) = 10.3$. Protein contributed $6.4 \times 1.54 = 9.9$ gm. of glucose. This experiment, therefore, does not furnish any evidence for the conversion of fatty acids to carbohydrates, since protein may have been the source of the extra glucose eliminated under the influence of epinephrine.

TABLE VI.

A.

Dog H, weight 6.8 kilos. Food and insulin discontinued June 13, 9.00 a.m.

Date.	Period.	Glucose.	Nitrogen.	D:N	R.Q.	O_2 per kilo per hr.	Calories per kilo per 24 hrs.	Remarks.
1927		*gm.*	*gm.*			*cc.*		
June 15, 12 hrs.	First 12 hr., 3rd day.	8.75	0.81	10.8	0.704	873	98.20*	
June 16, 12 hrs.	Second 12 hr., 3rd day.	9.27	2.10	4.4				
June 16–17, 24 hrs.	4th day.	28.8	5.74	5.0	0.700	925	104.0†	8 doses of 1 cc. 1:1000 adrenalin given every 3 hrs. over 24 hr. period.
June 17, 12 hrs.	First 12 hr., 5th day.	5.84	3.00	1.9				

B.

Dog H, weight 6.8 kilos. Food and insulin discontinued July 2, 9.00 a.m.

Date.	Period.	Glucose.	Nitrogen.	D:N	Phosphates.	R.Q.	O_2 per kilo per hr.	Calories per kilo per day.	Remarks.
1927		*gm.*	*gm.*		*mg.*		*cc.*		
July 4, 12 hrs.	First 12 hr., 3rd day.	31.6	1.62	19.5	258	0.632*	775	87.0	
July 5, 12 hrs.	Second 12 hr., 3rd day.	16.2	1.23	13.3	201				
July 5–6, 24 hrs.	4th day.	74.8	6.37	11.7	839	0.703†	894	101.0	8 doses of 1 cc. 1:1000 adrenalin given every 3 hrs. over 24 hr. period.

* Measured over first 3 hours of period.

† Measured over first 3 hours of period after first dose of adrenalin.

During the second period, the extra glucose eliminated under the influence of epinephrine was $74.8 - (2 \times 16.2) = 42.4$ gm. Protein contributed $3.91 \times 6.4 = 25.02$ gm. of glucose. Liver glycogen would account for $\frac{0.35 \times 340}{100} = 1.2$ gm. and glycerol for $\frac{6.8 \times 101}{10 \times 9.4} = 7.31$ gm. of glucose. Glucose derived from fatty acids was, therefore, $42.4 - (25.02 + 1.20 + 7.31) = 8.9$ gm.

Coincident with the rise in urinary glucose following the injection of epinephrine the inorganic phosphate excretion also rose. This increased excretion of phosphates persisted even after the epinephrine injections were discontinued. Thus, in Dog F (Table I), the excretion of inorganic phosphates during the epinephrine period was 340 mg., whereas during the previous 12 hours 177 mg. were excreted. Again, in Dog J (Table II), 470 mg. of phosphates were eliminated during the epinephrine period, whereas, during the previous and following 12 hour periods 203 and 480 mg., respectively, were excreted. In Dog I (Table V) the increase in the phosphate excretion did not occur during the epinephrine period, but during the following 24 hours.

Measurement of the respiratory exchange was carried out for a few hours in only two of the foregoing observations in order to obtain data which could be used in all of them for the purpose of calculating the amount of glycerol being metabolized. In this observation the R.Q. rose during the hour immediately following the injection of epinephrine but fell back to below the basal level during the succeeding hour. This rise in R.Q. is rather the exception than the rule following the injection of epinephrine (Soskin, 1927, *b*) and when it does occur is always temporary, being probably due to the hyperpnea and consequent blowing off of CO_2 which epinephrine causes in some animals. The general effect of epinephrine given to depancreatized dogs is, if anything, to depress the quotient.

Since the manuscript for this paper was submitted for publication we have carried out another observation in which the animal was kept in the respiratory cabinet for nearly 11 hours with occasional interruptions for the purpose of injecting epinephrine. The urine was also collected for analysis. The results are shown in Table VII, A.

TABLE VII.

A.

Dog K, weight 6.69 kilos. Depancreatized. Last meal and insulin Dec. 19, 9 a.m.

Date.	Period of fasting.	Glucose.	Nitrogen.	Remarks.
		gm.	gm.	
Dec. 22	Second 12 hr., 3rd day.	19.6	1.90	
" 22	First 12 " 4th "	12.7	1.54	
" 23	Second 12 " 4th "	9.70	1.46	
" 23	First 12 " 5th "	24.7	2.23	1 cc. epinephrine 1:1000 every 3 hrs. of this period.

B.

Date.	Time.	Procedure.	O_2	R.Q.	Remarks.
1927			cc.		
Dec. 22	3 hrs.	Control period.	6798	0.704	Animal quiet throughout.
Dec. 23	9.20 a.m.	1 cc. epinephrine 1:1000.			Beginning of first 12 hrs. of 4th day.
	9.45 a.m.–10.45 a.m.		6988	0.857	Much excited, barking and moving about.
	10.45 a.m.–11.45 a.m.		5810	0.727	Restless.
	11.45 a.m.–12.15 p.m.		2824	0.672	"
	12.17 p.m.	1 cc. epinephrine 1:1000.			
	12.39 p.m.–1.39 p.m.		5447	0.675	Mainly quiet, urinated.
	1.39 p.m.–2.39 p.m.		5769	0.673	More restless.
	2.39 p.m.–3.09 p.m.		2611	0.668	Quiet.
	3.10 p.m.	1 cc. epinephrine 1:1000.			
	3.37 p.m.–4.37 p.m.		5140	0.664	Quiet, urinated.
	4.37 p.m.–5.37 p.m.		4642	0.694	Sleeping.

TABLE VII—*Concluded.*

Date.	Time.	Procedure.	O_2	R.Q.	Remarks.
			cc.		
Dec. 23	5.37 p.m.–6.07 p.m.		2390	0.673	Sleeping, urinated.
	6.09 p.m.	1 cc. epinephrine 1:1000.			
	6.30 p.m.–7.30 p.m.		4447	0.691	Quiet.
	7.30 p.m.–8.30 p.m.		4547	0.673	"

From the results given in Table VII the following calculations may be made.

Extra glucose to be accounted for, $24.7 - 9.7 = 15.0$ gm.

Extra nitrogen eliminated, $2.23 - 1.46 = 0.77$ gm.

Maximum amount of glucose derivable from protein, $0.77 \times 6.4 = 4.9$ gm.

Weight of liver of this animal was 300 gm. ∴ glucose derivable from liver glycogen $= \frac{0.35 \times 300}{100} = 1.0$ gm.

Oxygen consumption for $9\frac{1}{2}$ hours during which epinephrine was given, 50.615 cc. ∴ oxygen consumption for 12 hour period = 64 liters.

Glucose derivable from glycerol assuming that nothing but fat was burned $= \frac{64.0 \times 4.7}{9.4 \times 10} = 3.2$ gm.

Total glucose accounted for by liver glycogen, glycerol, and protein = $4.9 + 1.0 + 3.2 = 9.1$ gm.

Glucose excreted unaccounted for, $15.0 - 9.1 = 5.9$ gm.

The R.Q. for all the periods of observation omitting the first, during which the animal was undoubtedly blowing off CO_2, is: $\frac{CO_2}{O_2} = \frac{29.782}{43.627} = 0.682$. We may correct this for the O_2 necessary to convert fatty acid into glucose, as suggested by Lusk, as follows: 0.465 gm. of O_2 required to produce 1 gm. of glucose from fatty acid. Therefore, to produce 4.2 gm. of glucose (the extra amount excreted during $8\frac{1}{2}$ hours) 1.95 gm. of O_2 or 1.37 liters are required and the corrected R.Q. is $\frac{29.782}{43.627 - 1.37} = \frac{29.782}{42.257} = 0.704$. If one should include the 1st hour, the R.Q. for the $9\frac{1}{2}$ hours is 0.707 or,

after allowing for the O_2 required for gluconeogenesis during this period (4.7 gm.) $\frac{35.771}{50.615 - 1.53} = \frac{35.771}{49.085} = 0.728$. This R.Q. is not uncommonly observed in depancreatized dogs which are being fed on food containing no carbohydrates (*cf.* also Hédon, 1926).

DISCUSSION.

The results of this investigation show *beyond doubt* that when epinephrine is injected at intervals into depancreatized dogs from which insulin and food have been withheld for 3 days, the excretion of glucose in the urine may increase far beyond what can be accounted for from preformed carbohydrate, protein, and glycerol. This result can be explained only in one way; namely, that the extra glucose is derived from fatty acid. It confirms the results previously obtained, though much less strikingly, by Eppinger, Falta, and Rudinger (1908), whose work, however, has not been universally accepted, especially since Ringer (1910), working with phlorhizinized dogs, could demonstrate no change in the D:N ratio following the administration of epinephrine. The less pronounced increase in glucose excretion observed by Eppinger *et al.* as compared with that observed by us, is probably to be accounted for partly by their not having allowed sufficient time for the collection of urine following the injection of epinephrine and partly by their having used recently depancreatized animals, which were in a much less favorable state of bodily nutrition than the insulin-treated animals employed by us. The failure of Ringer to confirm Eppinger's results is no doubt to be accounted for by his use of phlorhizinized instead of depancreatized animals. A fasted phlorhizinized animal is not a diabetic animal in the strict sense, for it still possesses its pancreatic islet tissue and it does not exhibit hyperglycemia. When epinephrine is injected into such an animal it no doubt tends to raise the blood sugar by exciting glycogenolysis,—or if no glycogen remains, by exciting gluconeogenesis,—and we may suppose that the increased blood sugar then stimulates the islet tissue so as to bring about a greater internal secretion of insulin which, of course, acts antagonistically to the epinephrine and so lowers the blood sugar. In depancreatized animals, on the other hand, hyperglycemia cannot stimulate the secretion of insu-

lin, so that epinephrine continues to excite gluconeogenesis. In the present investigation epinephrine was injected over a sufficient period of time so that all the extra glucose might be excreted, and in some of the experiments this period extended for 24 hours. This removes Ringer's objection with regard to the Eppinger *et al.* experiments, that the period during which the glucose excretion was observed following epinephrine was too short. In approximately one-half of our animals epinephrine also caused the excretion of nitrogen to be increased; in the other half the nitrogen either remained unchanged, or decreased. Even in those cases of the present series of observations in which a decided increase in nitrogen excretion occurred, it was far from sufficient to account for more than a small fraction of the extra glucose as coming from protein.

The fact that epinephrine causes a marked increase in the excretion of phosphates in the completely depancreatized dog at the same time that it stimulates the process of gluconeogenesis from fat, adds further evidence that there is a close relationship existing between the metabolism of carbohydrates and that of phosphorus (Fiske, 1920; Wigglesworth, Woodrow, Smith, and Winter, 1923; Winter and Smith, 1924; Sokhey and Allan, 1924). It is of interest to recall here that glucose injections in normal animals, like insulin injections, lower the phosphates of the blood and urine, while on the other hand, glucose fails to have any similar effect when injected into depancreatized animals (Bolliger and Hartman, 1925; Markowitz, 1926). This indicates that glucose acts by stimulating the internal secretion of insulin. But such cannot be the mechanism by which epinephrine acts, since as the present results show, it still affects the phosphates in depancreatized animals. Both epinephrine and insulin, therefore, act directly on the intermediary metabolic process which involves glucose and phosphorus, but it remains to be found out (by careful studies of the time relationships of the effects produced) whether they both act in the same way.

Although, in the light of previous observations by one of us (Chaikoff, 1927), our data with regard to the behavior of the ketone bodies are insufficient to warrant any conclusions as to whether epinephrine causes these to increase, this seems probable, and if it should prove to be the case, evidence in support of the

belief that a direct relationship exists between glucose, ketone bodies, and phosphorus in intermediary metabolism would be afforded. Further evidence for this belief is the fact that the injection of insulin into fasting depancreatized dogs causes the percentage of all these, namely glucose, phosphates, and ketone bodies, to fall at a parallel rate in the blood (Chaikoff, Macleod, *et al.*, 1925). These observations support the view that glucose is derived from fatty acid, and that the ketone bodies are by-products of this process. It is interesting further to consider the possibility that a phosphorized fat is the compound which is immediately concerned in the gluconeogenic process, which would account for the change in phosphorus accompanying those of glucose and ketone bodies.

Whatever may be the exact mechanism involved, there can be no doubt that injection of epinephrine into well nourished, depancreatized dogs brings about a conversion of fatty acid into glucose. We know of no evidence which would indicate that a similar conversion is not also capable of occurring in normal animals, and that it is in part responsible for the excessive sugar production in diabetes. According to this view, the low R.Q. observed in complete diabetes is explained not, as is usual, by supposing that fat exclusively is being oxidized, but by its representing the average of a very low quotient dependent on the absorption of oxygen to convert the methyl groups of fatty acid into the alcohol groups of carbohydrate on the one hand, and a quotient of unity due to carbohydrate combustion, on the other.

As to how epinephrine acts, in stimulating sugar formation from fat, little that is definite is known, but the available evidence would seem to indicate that it does so indirectly by causing the liver glycogen to disappear. When this occurs in depancreatized animals, the ketone excretion increases very rapidly (Chaikoff, 1927) and insulin immediately checks this excretion *pari passu* with that of glucose.

CONCLUSIONS.

Repeated injections of epinephrine into depancreatized dogs on the 3rd and 4th days after the withdrawal of insulin and food cause the excretion of glucose in the urine to rise far in excess of the amount which can be accounted for as derived from the glycogen stores of the liver, from protein, and from glycerol.

On the improbable supposition that the glycogen of the muscles can also be a source of the extra sugar, there is still an excess to be accounted for in two of the experiments.

The extra sugar must be derived from fatty acid.

The excretion of phosphorus by the urine is greatly increased during the periods of injection of epinephrine.

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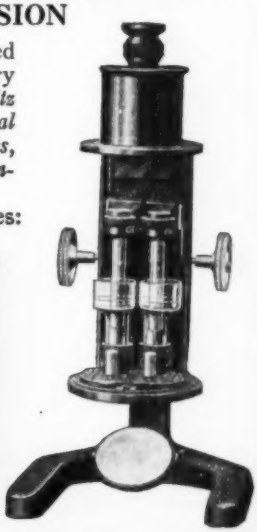

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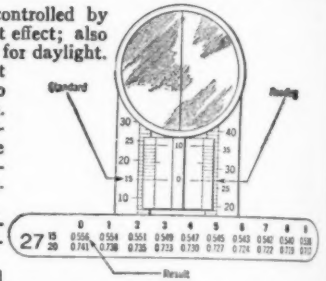

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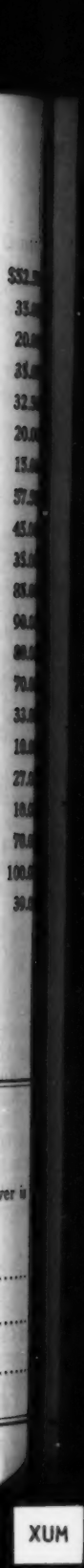

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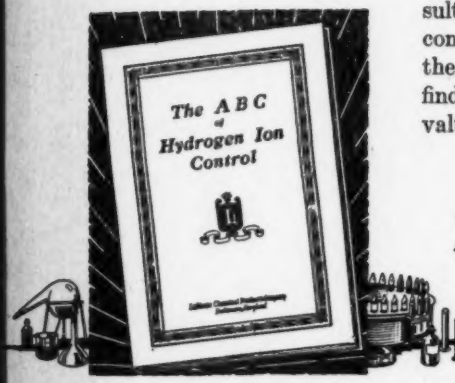

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